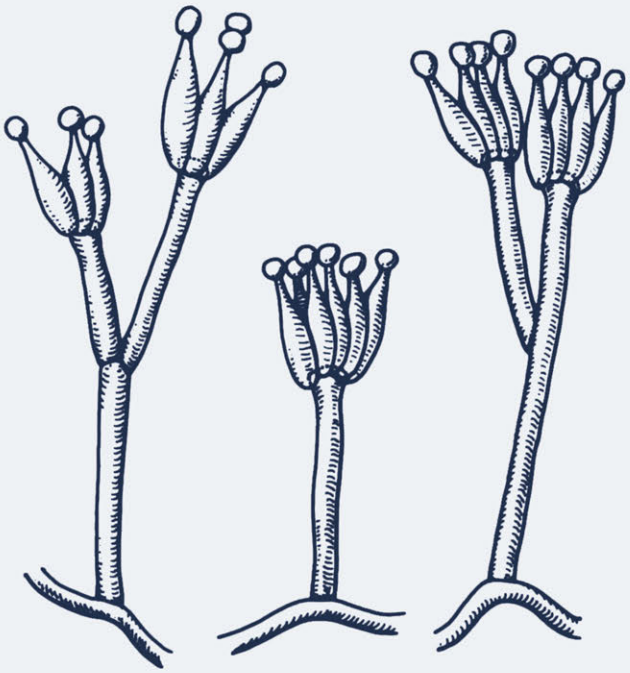
BIODETERIORATION RESEARCH 3

Mycotoxins, Biotoxins, Wood Decay, Air Quality, Cultural Properties, General Biodeterioration, and Degradation



Edited by
Gerald C. Llewellyn
and
Charles E. O'Rear

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PREFACE

Once again the present volume contains the majority of the papers presented at the Third Pan-American Biodeterioration Society Meeting held at The George Washington University, Washington, D.C., USA, on August 3, 4, 5, and 6, 1989. The sponsors for this symposium included The George Washington University, The Smithsonian Institution, The Virginia Department of Health, The University of Connecticut, The National Institute for Occupational Safety and Health, Clark Atlanta University, Ball State University, the U.S. Naval Research Laboratory, the Agriculture Research Service/U.S. Department of Agriculture, the University of Georgia, the Metropolitan Museum of Art, Morehouse College, the University of Texas at Houston, North Carolina State University, the U.S. Food and Drug Administration, and the Forest Service/U.S. Department of Agriculture.

The program was developed by members of the Program, Planning, and Organizing Committee. Leading scientists in specific topic areas were invited. Also we accepted contributed papers from individuals and laboratories actively involved in relevant areas of research and study. The participation of internationally established scientists was encouraged. The Society (PABS) tried to ensure that the program reflected current developments, informed reviews, embryonic and developing areas, and critical assessment for several aspects of the present state of knowledge as it relates to the major sections of the proceedings. Obviously, not all aspects of biodeterioration or biodegradation are represented.

All the papers included herein underwent scientific and technical peer review. Some papers do not appear in the volume because they were judged not suitable for publication or failed to meet specific deadlines or format requirements. Included also are several papers that were either not fully presented at the meeting, arrived after the meeting program was established, or were considered appropriate to complement the presented program topics. The order and organization of the

contributions vary somewhat from that of the actual meeting session. Program speaker papers are grouped with their appropriate topical section.

This book is the result of the efforts of the Program, Planning, and Organizing Committee, section organizers, PABS co-program chairmen, session chairpersons, authors, and those behind the scene assistants listed in Acknowledgments and Appreciations. The assistance provided by the many scientists who provided us with timely reviews of the manuscripts was a significant factor, allowing for the rapid publication of the symposium.

This publication is considered to be a general contribution to the broad sciences of biodeterioration, degradation, and deterioration in general. There has been maintained an increased emphasis upon wood decay in this, the third meeting. An expanded, additional emphasis in indoor air quality topics was included in the program for the first time. The largest aspect of the meeting continued to be associated with mycotoxins. In this topic, the editors continue to recognize their bias as well as the historical role that fungal toxins have played in the original development of our parent society, The International Biodeterioration Society in the United Kingdom.

The international aspect of this book and meeting is supported by authors, program speakers, and/or other participants representing six nations. Participants primarily represented North America, therefore quite appropriately complementing PABS, the Pan-American name sake. The European and Far East representation must be recognized also, as is evident in the following overall listing of countries represented: Sweden, Canada, Yugoslavia, Republic of Singapore, Indonesia, and the United States of America.

We believe these proceedings, especially since they have been reviewed and edited and include work from beyond the western hemisphere and Pan-America, will be of substantial value to a broad range of scientists.

The Editors

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The cover illustrates Penicillium citrinum.

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INTRODUCTION AND OVERVIEW

The Role Of Biodeterioration In Agriculture, Forestry, Agronomy, Cultural Properties, Economics, And Public Health

Biodeterioration is often a misnomer, in that we tend to use biodegradation, deterioration, degradation, pollution, decay and other terms as synonyms. As authors and editors of this proceedings we are also guilty of a very "loose" application of the definition. For example, numerous examples of health-related issues have crept into this editon via papers relevant to the reduced quality of human food, animal feed, parasites, water quality, air quality, and diseases in general. But the mainstays of biodeterioration are also present and they support the basic definition as follows: Biodeterioration is an undesirable change caused by microorganisms. Generally fungi and bacteria are prime deteriogens, but other organisms such as plants, invertebrates, birds, and rodents are often "legally" included. Not to be confused but often excluded by the "purist", is biodegradation. The latter is often considered as a planned, controlled, and purposeful breakdown or alteration due to organisms. There is no doubt that this text continues to apply a very broad and multidisciplinary interpretation to biodeterioration.

The information contained herein is much more important than debating a definition. The papers reported touch every aspect of our lives including building materials, water, food, medicine, clothing, and art objects. Disciplines involved begin with microbiology, mycology and biochemistry and extend to economics, agriculture, forestry, agronomy, industrial hygiene, human and veterinary health, epidemiology, forensic science, toxicology, chemistry, physics, soil biology and chemistry, conservation in museums, cultural materials, and economics.

The major biodeteriogenic topics discussed in this

proceeding concerns the areas of wood decay, mycotoxins, organisms affecting air and water quality, alteration and degradation of enzymes, effects of toxins, microorganisms, and xenobiotics upon cellular systems, organisms affecting food and feed quality, deterioration of man-made fibers in soil, biocides and statue restoration, controlling biodeterioration in museums, and wood preservation.

Although there continues to be valued classical-type biodeterioration studies included in the chapters, modern and recently developed techniques are also an integral part. For example, there is included in this edition a broad-spectral application of immunological techniques. Also models have been developed applying recent biotechnology developments. Methodological progress is reported for numerous quantitative-and qualitative-oriented studies. They range from a basic scientific and research laboratory approach to developments that are applicable to the regulatory process. From a biodeterioration standpoint, a common thread in many of the articles and all the sectional topics is the effort to predict, detect, and control undesired effects of microorganisms. Prevention of biodeterioration is the engine that drives the topic.

The proceedings, with its five major sectional topics relevant to wood decay, mycotoxins, air quality, and cultural properties, including general biodeterioration, provides the basic organizational format. Biodeterioration and biodegradation, with its inherent multi-disciplinary nature, provides the basis for substantial sharing and exchanging of knowledge. It is of the utmost importance that the information in these areas be communicated so that scientists involved in sub-disciplines may mutually benefit. So, presented in this the third edition, are recent reports and review articles by biodeteriologists, as they relate to agriculture, forestry, conservation and preservation, toxicology, environmental biology, and human health.

Gerald C. Llewellyn, Ph.D.
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SECTION I

THE ROLES OF MYCOTOXINS AS BIODETERIOGENS

THE ROLES OF MYCOTOXINS AS BIODETERIOGENS

INTRODUCTION

There are two parts to this section: Part A entitled, "Mycotoxicoses, In vitro, In vivo: Conjecture - Hypothesis - Validation" and Part B entitled, "Mycotoxins: Their Detection, Occurrence, Interaction, and Ecology" . These two parts, each having its own introduction, are the basis by which the symposia were organized. Most of the contributed papers have been included in Part B, because they were relevant to the general topic covered. Therefore Part B is a more generalized representation of the actual symposium and speakers at the meeting. Some papers were repositioned and placed into the most appropriate part or another section, based on their content. There is no doubt that both the very specialized Part A and the very general Part B, both contribute to recent developments in mycotoxinology and mycotoxicology as they relate directly and indirectly to biodeterioration.

It was the objective of the organizers to provide an opportunity to include traditional studies relating mycotoxins to biodeterioration as well as providing innovative and controversial reports such as those in Part A.

There is little doubt that the topics covered in both Part A and Part B show the diversity and interdisciplinary nature of mycotoxicology and mycotoxinology. Studies ranged from interests in mycology, to toxin production, to responses in animals, and to reviews on specific toxins. In reality, mycotoxins affect not only the farmer but the consumer as well. There are reports discussing the molecular, cellular, and organism levels. The scientific disciplines range from chemistry, to mycotoxicology, to biochemistry, to analytical chemistry, to toxicology, to organic chemistry, to economics, to veterinary science, and to human health.

Based on these papers in both Part A and Part B, there is continued evidence that this topical area deserves both research and regulatory support in an effort to control aspects of biodeterioration which affects the health and economics of a variety of citizens in most countries on earth. Areas that need special emphasis continue to be the identification of the problem, control, prevention, and application of new technology. It is expected that research needs in this area will continue well into the 21st century.

Recognizing that mycotoxicology and/or mycotoxinology are approximately 30 years of age at the present time, it is only necessary to study the reports presented by the scientists in the following two sections as they share their developments and progress as it is relevant to biodeterioration, biodeteriogation and toxicology, to illustrate the chronology which continues from the early 1960's. It is within the realm of reasonable expectation that the role of biodeterioration in mycotoxicology/mycotoxinology will continue at least for another three decades.

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PART A

MYCOTOXICOSES, *IN VITRO* AND *IN VIVO*: CONJECTURE–HYPOTHESIS–VALIDATION

MYCOTOXICOSES, *IN VITRO* AND *IN VIVO*: CONJECTURE-HYPOTHESIS-VALIDATION

INTRODUCTION

It is beyond question that a multitude of fungal metabolites are absorbed by animals and man on a regular basis. The significance of this fact to animal and human health is obvious only when accumulation results in acute toxicity. Realistically, however, acute toxicity is the exception rather than the rule and the effects of low level consumption, inhalation or percutaneous absorption of these biologically active fungal metabolites are for the most part unknown. All researchers who work with fungal metabolites, frequently ponder this great "unknown". Occasionally, a conjecture or hypothesis will appear in print, buried in the recesses of the Discussion, but the most frequent location for conjecture and hypothesis is in offices, hallways, restaurants and pubs, far from the reach of even the most powerful computerized information retrieval system. The goal of this session of the Third Meeting of the Pan-American Biodeterioration Society was to provide a forum in which presenters and discussion panel members were encouraged to extrapolate from what is known experimentally, in vitro and in vivo, to what is not known. For example, to attempt to connect logically the results of in vitro work to the in vivo situation with special reference to subacute levels of exposure. Presenters and panel members were also encouraged to present testable hypothesis for revealing the potential of fungal metabolites to affect biological function with chronic exposure and at environmentally realistic levels of contamination of foods and feeds. It was fitting that this session was part of a society whose primary concern is the biological deterioration of matter since the ultimate matter subject to biological degradation must be that of our own physical being.

There were six papers presented and following each paper there was a discussion which was led by the invited panel comprised of Drs. Bruce Jarvis and George Bean of the University of Maryland, and Dr. Winston Hagler of North Carolina State University. Each mycotoxin covered, was

the subject of some recent controversy. The discussion following the paper on fusarin C focused on the fact that this mycotoxin is probably not involved in either leukoencephalomalacia or esophageal cancer, as once suspected. The use of bioassays with animal cell culture was proposed as the ideal approach for identifying commodities containing Fusarium moniliforme toxins. With ochratoxin A, the relationship between endemic Balkan nephropathy and this toxin was the main subject of discussion. The fact that the kidney of humans suffering from Balkan endemic nephropathy is usually small where as animals intoxicated with ochratoxin A have enlarged kidneys was questioned. The recent NTP report that ochratoxin A is carcinogenic in rats was also discussed. It was pointed out that while ochratoxin is not a problem in the United States, it is a problem in Canada and parts of Europe. The discussion on patulin centered on the fact that patulin is still considered by some to be a threat to consumers even though in vivo toxicity is quite low. The carcinogenic potential of patulin and its potential as a peroxidative agent was also discussed. There was considerable discussion following the paper on cyclopiazonic acid. It was suggested that the conflicting results in toxicity studies with rats might be due to genotypic and phenotypic differences. This suggestion was based on the finding that the toxicity of aflatoxin B_1 in broiler chickens is much lower today than it was several years ago. The possibility that cyclopiazonic acid may be converted to iso-cyclopiazonic acid or a less toxic tautomeric form in bicarbonate buffer was also discussed.

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Mycotoxicoses, *In Vitro* and *In Vivo*: Conjecture-Hypothesis-Validation

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INTRODUCTION

The ultimate goal of food safety research is to ensure the quality and safety of the food supply. This includes the safety of both animal feeds and human foods. Within the concept of "biodeterioration", the impairment of human and animal health through the consumption of fungal metabolites in the food supply seems an appropriate, if not the ultimate, subject for study. Our health, and that of the animals upon which we depend, is clearly an area of great public concern. This concern creates the social and political force which sustains the monetary support of all professional scientists who study the biological activity of fungal metabolites which contaminate foods and feeds. Unfortunately, the public's concern for food and feed safety is sometimes forgotten or avoided by scientists who enjoy doing experimental research. Few would disagree, that solving basic problems is important, however, the consumer's concern is the applied problem -food and feed safety. In the field of mycotoxicology, it is difficult to maintain focus on this applied problem because the chemical and biological diversity of the subject matter reveals an infinite number of exciting and rewarding areas for basic research, while the epidemiological database that supports the hypothesis that fungal toxins in the food supply are a threat to American consumers is extremely limited or non-existent (Food and Chemical News, 1986). The animal risks are more apparent though often not well documented. For these reasons it is important that we, as research scientists, occasionally step back and evaluate where we are in terms of identifying those fungal metabolites which might pose a significant risk to human or animal health.

EXTRAPOLATION FROM IN VITRO TO IN VIVO

Whereas, in the laboratory it is relatively easy to identify metabolites which are toxic, extrapolating these results to human and

animal health is quite difficult. The in vitro and in vivo toxicity experiments which toxicologists conduct in the laboratory are usually oversimplifications of the real world situation. Relating the results from in vitro studies to in vivo, and relating this to food and feed safety is a speculative art. For example, in vitro studies in my laboratory have shown that cyclopiazonic acid is a potent inhibitor of calcium-dependent ATPase activity, calcium accumulation and calcium efflux in rat sarcoplasmic reticulum vesicles (Goeger et al., 1988; Goeger and Riley, 1989). We also know that cyclopiazonic acid causes alterations in calcium homeostasis in cultured muscle cells (Riley et al., 1989) and that concurrent with this there is an increased intracellular electronegativity that is associated with a fixed charge alteration which appears to be restricted to the cytoplasmic surface of the plasma membrane (Riley et al., 1986 and 1987). Demonstrating whether or not the fixed charge alteration observed in cells is causally related to the alterations in calcium homeostasis will be experimentally quite difficult as will be relating the fixed charge alterations in muscle cells to the effects on calcium transport in sarcoplasmic reticulum vesicles. Nonetheless, there is a theoretical basis for mechanistically connecting these phenomena (Krueger, 1989). A more arduous task will be equating the in vitro biological activity with the in vivo toxicity of cyclopiazonic acid. Here again, there is a theoretical basis for equating alterations in calcium homeostasis to toxicity (Komulainen and Bondy, 1988; Trump et al., 1981) and there exists data from in vivo studies which could be explained due to cyclopiazonic acid-induced alterations in calcium dependent processes (Peden, 1989). However, the most difficult task will be relating the in vitro biological activity to food and feed safety. At this point it seems unlikely that the safety of American consumers will ever be threatened by cyclopiazonic acid in the food supply. But are there specific sub-populations which may be at risk? Would in vivo inhibition of calcium-dependent ATPase activity or altered calcium flux at the cellular level impair the functional capacity of certain individuals under specific physiological conditions? Are there animal populations at risk? The in vitro data cannot stand by itself to demonstrate risk, but it can point the way to new experiments which may identify populations at risk and thus improve the safety of the food supply by establishing controllable factors for those who are identified as being at risk.

RISK ASSESSMENT

This past year the intensity of the food safety debate has increased

and the risk associated with natural products which contaminate foods and feeds has received considerable attention (Kilman, 1989; Ames and Gold, 1989). An interesting aspect of the debate is illustrated in the discussion over the human carcinogenicity of aflatoxins. The accumulated data is, for the most part, accessible and the studies which have been conducted are relatively straight forward with results easily comprehended by most biological researchers. Nonetheless, the same data is often interpreted quite differently by equally competent scientists. Based on the same data, some argue that the evidence suggests that AFB_1 is not a carcinogen in man (Stoloff as quoted by Food and Chemical News, 1989a), while others have concluded that there is sufficient evidence of carcinogenicity in humans (International Agency for Research on Cancer, 1987). While this debate on the interpretation of the data continues, the National Toxicology Program has proposed the addition of "Aflatoxins" (not just AFB_1) to the list of known human carcinogens in the Sixth Annual Report on Carcinogens (Food and Chemical News, 1989b). Interpreting existing data can be difficult and divisive, however even more complicated situations can be envisioned. For example, it has been suggested that one consequence of reduced use of Alar could be higher levels of the mycotoxin patulin in apple juice (Ames and Gold, 1989). Ames and Gold (1989) state that the "carcinogenicity of patulin has not been adequately examined" and cite the International Agency for Research on Cancer (1986) as support for this statement. The actual IARC evaluation of patulin is:

> "There is inadequate evidence for the carcinogenicity of patulin in experimental animals. No evaluation could be made of the carcinogenicity of patulin to humans" (International Agency for Research on Cancer, 1986).

This serves to illustrate that even though the IARC conclusion is clearly stated, there is room for interpretation. The nature of the interpretation depends on perspective of the interpreter. For example, those in fear of future litigations will take the conservative position. The Material Safety Data Sheet provided by Sigma Chemical Company when purchasing patulin, indicates that patulin is a suspected animal carcinogen and on the vial in which it is supplied is the statement "May cause cancer". Ames and Gold (1989), and Sigma Chemical Co., apparently takes a more conservative view than some other U.S. scientists who recently concluded that "...on the basis of occurrence and/or toxicity, patulin.....does not appear to be a real concern" (Jelinek et al., 1989). The consequence of disparate opinion is that unless they are experts on a particular compound, often even scientists must chose sides on subjective

rather than objective grounds. At the same time new mycotoxins are being discovered. These newly discovered fungal metabolites will warrant serious study if they are found to contaminate foods or feeds. For example, the causative agent (fumonisin B_1) of leucoencephelomalcia (LEM) has recently been identified (Marasas et al., 1988a) and it has been found in corn associated with LEM in the United States (Voss et al., 1989). Fumonisin B1 deserves attention because it is a potential animal carcinogen (Gelderblom et al., 1988a and 1988b) and it is produced by Fusarium moniliforme, one of the most prevalent fungi found on food and feed commodities such as corn (Marasas et al., 1984). The presence of F. moniliforme on corn has been associated with human cancers (Marasas et al., 1988b). Twenty five years of intense scientific research on aflatoxins has not resulted in a consensus of scientific opinion on the carcinogenicity risk to humans although it is agreed that AFB_1 is a potent hepatotoxin. It will be interesting to see where we are twenty five years from now with the fumonisins.

It is beyond question that a multitude of fungal metabolites are absorbed by animals and man on a regular basis. The significance of this fact to animal and human health is usually obvious only when accumulation results in acute toxicity or carcinogenicity. Interestingly, of all the myriad mycotoxins which contaminate foods and feeds, only aflatoxins are considered by the IARC and NTP to be human carcinogens (Table 1). IARC groups sterigmatocystin as a possible human carcinogen and NTP has proposed ochratoxin A for inclusion as "reasonably anticipated to be a carcinogen" (Food and Chemical News, 1989b). It has been estimated that the cancer risk associated with mycotoxins in human foods is 7.61×10^{-8} (Scheuplein, 1990) the lowest risk estimate of the 7 food categories, containing carcinogenic substances, that were compared. Realistically, however, acute toxicity and carcinogenicity are the exceptions rather than the rule and the effects of low level consumption, inhalation or percutaneous absorption of these biologically active fungal metabolites are for the most part unknown. All researchers who work with fungal metabolites, frequently speculate on the potential of fungal metabolites in the food and feed supply to alter immune response, reproduction, development, behavior, renal and liver function, etc., in high risk human and animal populations. For example, the effects of zearalenone on pregnant women and young children, ochratoxin on renal function in people with kidney disorders, cyclopiazonic acid on individuals with muscle disease, tricothecenes on individuals with immunological disorders, etc. Unfortunately, these speculations seldom appear in the accessible

Table 1. Summary of the International Agency for Research on Cancer (IARC) Evaluation of Carcinogenic Risk for Fungal Metabolites Found in Foods [a].

Compound	Degree of Evidence for Carcinogenicity[b]		Overall Evaluation[c]
	Human	Animal	
Aflatoxins	S	S	1
Citrinin	ND	L	3
Cyclochlorotine	ND	I	3
Fusarenon X	ND	I	3
Luteoskyrin	ND	L	3
Ochratoxin A	I	L	3
Patulin	ND	I	3
Penicillic acid	ND	L	3
Sterigmatocystin	ND	S	2B
T2-tricothecene	ND	I	3
Zearalenone	ND	L	3

[a]Excerpted from Table 1 of the IARC (1987) overall evaluation of carcinogenicity update of IARC monographs 1 to 42.

[b]ND, no adequate data; I, inadequate evidence; L, limited evidence; S, sufficient evidence. Detailed definition of terms can be found in the Preamble to IARC, 1987.

[c]Overall evaluation 1, "carcinogenic to humans"; 2B, "possibly carcinogenic to humans"; 3, "not classifiable as to their carcinogenicity to humans".

scientific literature, although they occasionally turn up in the popular press.

It is often politically and professionally dangerous to extrapolate the cause and effect relationships revealed from controlled experiments to diseases which are clearly multifactorial. The legal and moral pitfalls and economic damage that can be done are well illustrated by the recent

series of events related to the occurrence of Alar in apples (Koshland, 1989; Groth, 1989), aflatoxins in corn (Kilman, 1989) and even toxins as yet to be identified (Marshall, 1983). It is my opinion, and I believe that of many other research scientists, risk assessment is avoided because it appears to be more political than scientific. Nonetheless, it is better that the discussion be initiated by researchers in a scientific forum rather than by celebrities speaking before congressional committees, an enraged citizenry going to the polls, or journalists in the popular press. As scientists we must be as open to being educated by the public as we are ready to educate. Edward Groth (1989) stated this need well in his letter to Science:

> "....we must listen to what people say about risks. It is not the size of the risk but its moral offensiveness that makes the public respond so strongly." When scientists are apathetic to public concern then "...the posture they strike makes scientists seem arrogant, insensitive, and unconcerned about things that matter a lot to average people. If such reactions predominate, both the quality of the public debate and the perception that science has helpful solutions will suffer grave damage".

At the same time it is important to remain cognizant of the fact that in a democracy, those who are recipients of public funds, "must respond to the picture of what is in the public mind, even if that picture is unrealistic" (Herbert Stein, as quoted in The Wilson Quarterly, Summer, 1989). It is in fact the unrealistic picture to which scientists must be most responsive. The following is excerpted from the April 5, 1989 cover letter which accompanied the the report of the 1988, Office of Scientific Public Affairs-organized, Food Safety Workshop (Greenburg, 1989):

> "...However, also apparent in the report is the scientists' frustration with the public misconceptions of risks associated with foods. These misconceptions give rise to the political pressures that force our food protection agencies to concentrate their resources on minor risks (pesticides and drug residues) rather than more important threats (microbiological and natural toxicants). The report emphasizes the responsibility of the scientific community to communicate the concept of risk more effectively to the public."

For basic researchers, the goal of communicating the concept of risk to the public is probably an unrealistic aspiration. However, within the realm of possibility, and perhaps the first step in the thousand mile journey to attain the former goal, is to communicate to each other our own concepts of risk based on our in vitro and in vivo studies.

THE GOAL OF THIS SESSION

The goal of this session of the Third Meeting of the Pan-American Biodeterioration Society is to provide a forum in which presenters and discussion panel members will be encouraged to extrapolate from what is known experimentally, *in vitro* and *in vivo*, to what is not known. For example, 1) to attempt to connect logically the results of *in vitro* work to the *in vivo* situation with special reference to subacute levels of exposure; 2) to attempt to explain those experimental paradoxes which confound our attempts to offer either objective or subjective risk assessment. Presenters and panel members will also be encouraged to present testable hypotheses for revealing the potential of fungal metabolites to affect biological function with chronic exposure and at environmentally realistic levels of contamination of foods and feeds. Each fungal metabolite to be discussed is the subject of some controversy. Ochratoxin A is of concern because it has been proposed for inclusion in the list of substances "reasonably anticipated to be carcinogens" (Sixth Annual Report on Carcinogens) and it has been implicated in human and animal nephropathy. Cyclopiazonic acid is of interest because it appears to be more prevalent than previously assumed (Jelinik et al. 1989), it has been implicated in human disease (Rao et al., 1985), and the results of several toxicity studies with rats are not in agreement (Purchase, 1971; van Rensburg, 1984; Morrissey et al., 1985; Voss et al., 1989). Patulin is of interest because, even though some scientists believe it to be of little concern (Jelinek et al., 1989), there are others who believe that patulin has not been adequately studied (Ames and Gold, 1989). T-2 toxin, and many tricothecenes, are potent immunomodulators. An important question is the role immunosuppressive mycotoxins play in animal disease and how they affect immunization programs designed to protect farm animals from common pathogens. Finally, fusarin C is of interest because it has been shown to be a potent mutagen in the Ame's test (Gelderblom, et al., 1983) and although it is not a carcinogen (Gelderblom et al., 1986), it is produced by the same isolates of *F. moniliforme* which produce the newly discovered, suspected animal carcinogen, fumonisin B1 (Bezuidenhout et al., 1988).

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Patulin: Mycotoxin or Fungal Metabolite? (Current State of Knowledge)

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INTRODUCTION

Following the discovery of penicillin by Fleming in 1929 a flurry of work ensued in which similar approaches were applied to the search for other antibiotics. One of the products of this search was the compound patulin, initially isolated from filtrates of a strain of Penicillium claviforme by Chain et al. (1942). The compound, originally referred to as clariformin and possessing potent antibacterial activity, was the subject of numerous chemical and biological studies soon after its isolation from a strain of Penicillium patulum Bainier and characterization by Birkinshaw et al. (1943). Elucidation of the structure was made by Woodward and Singh (1949). It has since been shown to be produced by several species of Aspergillus and Penicillium and at least one species of Byssochlamys, as reviewed by Ciegler (1977) and Stott and Bullerman (1975).

Patulin (Figure 1) has a furopyrone structure containing an alpha-beta unsaturated lactone system with the carbonyl group of the lactone conjugated to the double bonds. The double bonds thus become susceptible to nucleophilic attack additions as exemplified by the Michael addition reaction. Such addition reactions have been postulated to explain reactions between sulfhydryl-containing amino acids and patulin that result in apparent modifications in the toxin structure (Singh, 1967; Hofmann et al., 1971; Ciegler, 1977). However, the chemistry involving this polyketide lactone is apparently quite complex. For example, there are indications that the compound reacts not only with sulfhydryl groups but also with amino groups of amino acids and proteins under the right conditions (possibly also by addition reactions)

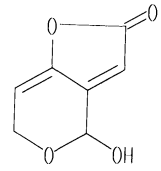

Figure 1. Chemical structure of patulin (4-hydroxy-4H-furol[3,2-c]pyran-2(6H)-one).

(Ashoor and Chu, 1973b; Ciegler, 1977; Lindroth and von Wright, 1978) and that ring opening of the hemiacetal group may allow for the formation of Schiff bases with the amino groups of free amino acids or proteins (Wallen et al., 1980). The carbonyl group of the lactone is also available for possible reaction with the amino groups of amino acids or proteins. Unfortunately very little work has been carried out to properly isolate and characterize the adducts of patulin. This will be vital in elucidating the mechanisms of the toxicity and reactivity of patulin in biological systems.

Patulin is quite soluble in aqueous solution; it absorbs UV light with a single peak at 276 nm (which decreases after reaction with SH groups) and is quite stable in acid solutions (between pH 3.3 and 6.3) and unstable in alkaline solutions (Stott and Bullerman, 1975). Its instability in some foods has been attributed to reaction with SH-containing compounds (Ciegler, 1977). Information concerning the levels of patulin reported in some foods or food products is summarized in Table 1.

Although patulin and/or strains of fungus known to produce the toxin under artificial conditions are found in a number and variety of food commodities, rotted apples and products prepared from apples are the principal foods that contain patulin in measurable quantities (Stott and Bullerman, 1975; Ciegler, 1977) and in which patulin is stable (Pohland and Allen, 1970; Lovett and Peeler, 1973). IARC (1986) reported levels of toxin from 0.001 to 1.2 ppm in apple juice and up to 100 ppm in rotted apples. Other reports indicate from 45 ppm in "organic apple cider" (Wilson and Nuovo, 1973) to 1000 ppm in apple sap (Brian et al., 1956). It can be calculated that for a 25-kg child drinking a liter of apple juice containing 0.052 ppm patulin, and assuming complete absorption (see

Table 1. Levels of Patulin in Some Foods and Food Products[a].

Food or Food Product	Concentration (ppm)
Apples (rotten)	0.2 - 100
Apple juice	0.001 - 1.2
Apple juice (USA)	0.044 - 0.309
Baby food (apples)	0.01 - 0.1
Bread (moldy)	0.02 - 0.3
Other juices	0.021 - 0.062

[a]Adapted from IARC (1986).

discussion later) and uniform distribution, a tissue or blood level of 1.35×10^{-8}M patulin could be obtained. However, if we use the extreme level of 45 ppm, a level of approximately 10^{-5}M is obtained for the theoretical concentration of the toxin and/or its metabolites in the body.

Toxicologic studies of patulin were started about 45 years ago; many of them involved the application of pharmacological systems, including the use of isolated organs and the intravenous (iv), intradermal, intramuscular, and intraperitoneal (ip) dosing of animals. In fact, a paper entitled "Pharmacological Studies of the Effects of Clavatin" (now recognized as one of the synonyms for patulin) by A. Schweitzer, one of the first in the series, was dated November 1946. Because of the potent antibiotic properties of patulin, an extensive amount of such work was carried out, but the conclusion of this particular paper, confirmed in part by others, was that patulin was too toxic to be considered for therapeutic use in humans. Information from many of these studies indicated that patulin, under the conditions employed, was capable of producing edema and congestion and hemorrhage in several organs (Katzman et al., 1944; Escoula et al., 1977). As to whether patulin should be categorized as a true mycotoxin or simply as a mold metabolite, consider the definition of mycotoxins proposed by Bennett (1987): "Mycotoxins are natural products produced by fungi that evoke a toxic response when introduced in low concentration to higher vertebrates and other animals

by a natural route." Bennet made no attempt to define low concentration, but according to the Registry of Toxic Effects of Chemical Substances (1985-86 edition) one criterion of a toxic substance is any chemical substance that has a median lethal dosage (LD_{50}) of less than 500 mg/kg of body weight when administered orally to rats. Of course the "natural" route for patulin in humans would be oral ingestion. Applying this criterion, patulin certainly meets the definition of a toxin and thus that of a mycotoxin. When considering in vitro toxicity, most toxicologists would consider effect levels of anything above one millimolar to be of little physiological signficance. Here too, patulin must be regarded as a substance with considerable biological activity.

Data presented in this review deal with the effects of patulin, almost exclusively but not entirely, on mammalian cells and in animals administered the toxin via the oral route. Although patulin can produce a variety of effects on cells at low concentrations, the in vivo data are insufficient and thus do not allow us to conclude that patulin is of no real concern, as has been stated recently (Jelinek et al., 1989), or to implicate it as a true and potent mycotoxin with potential risk to humans. Some human data available from the time when patulin was considered a candidate for treatment of human infections (including the common cold) indicate that 0.1 g of compound administered iv produced no apparent effects (De Rosnay et al., 1952) but given orally caused upper gastrointestinal symptoms in humans (De Rosnay et al., 1952; Freerksen and Bonicke, 1951). Patulin also produced dermal irritation when applied to the skin of humans (de Wit, 1946; Dalton, 1952), a result that may be related to the early finding that 0.2 mg of the toxin produced sarcoma in rats when injected subcutaneously twice a week for 64 weeks (Dickens and Jones, 1961). Patulin was also implicated as the etiologic agent in the mass death of many dairy cows in Japan in 1952 (Ciegler, 1977).

A collage of laboratory data is presented here to illustrate the types of effects patulin produces in vitro, the range of effect levels, and some of the effects produced in animals after oral administration.

IN VITRO EFFECTS

Growth and Morphology

An overview of the effects of patulin on various types of cells is shown in Table 2. Patulin is toxic to a wide variety of cells--bacteria,

Table 2. Toxicity of Patulin to Microorganisms and Mammalian Cells[a].

Microorganism or Mammlian Cell	Growth Inhibitory Concentration (M x 10^{-5})
Bacteria	
Gram negative	1.30 - 13.0
Gram positive	2.60 - 64.9
Fungi	2.14 - 194.8 (some not affected even at 649)
Protozoa	0.06 - 64.9
Mammalian cells	
Normal[b]	6.49 - 130.0
Cancer[c]	0.65 - 39.0

[a]Summarized from data in Singh (1967).

[b]Mouse leukocytes, normal rabbit corneal epithelium, chick heart, and fibroblasts.

[c]L-cells, Ehrlich carcinoma, mouse ascites tumor cells.

protozoa, or normal or transformed mammalian cells. Levels that affect growth are as low as 6 x 10^{-7}M for some protozoa or 6.5 µM for certain cancer cells, but some strains of fungi apparently resist the growth-inhibitory effects of patulin even at 6.5 mM. Levels of patulin effective for modifying growth or morphological characteristics of cells are summarized in Table 3. These cells are primarily of mammalian origin, but for comparison purposes effect levels for some microbial, chick embryo, and yeast cells are shown. The most sensitive system here was Chang liver cells in which growth was inhibited at 6.5 x 10^{-7}M, but there is a broad array of effects, including inhibition of mitosis in fibroblasts and osteoblasts of chick embryo cells, HeLa cells, Chinese hamster cells, and rodent fibroblasts (10^{-6}M to 5 x 10^{-5}M), disorganization of cytoplasmic microfilaments in hepatoma cells (3.0 x 10^{-5}M), cytoskeleton changes in renal epithelial cells (10^{-5} to 2.5 x 10^{-4}M) and cell volume increases of macrophages (5.0 x 10^{-5}M). Thus for these biological test systems the range of effect levels was about 10^{-4} to 10^{-7}M. Rihn et al. (1986) state that the effect of patulin on hepatoma cells resembles the effects seen for other cytoskeleton poisons like colchicine and cytochalasins.

Table 3. Effects of Patulin on In Vitro Systems. I. Morphological and Growth.

Reference	System	Effect or Assay	Effective Dose (M)
Rihn et al., 1986	Hepatoma cells	Disorganization of cytoplasmic microfilaments; loss of membrane microvilli; inhibition of mitosis	3.0×10^{-5}
Kawasaki et al., 1972	HeLa cells	Inhibition of cell growth, proliferation and cell cycle; pleomorphism of cytoplasm and nucleus	1.54×10^{-5}
Hinton et al., 1989	Renal epithelial	Blebbing of cells	10^{-5}
Schaeffer et al., 1975	Chang liver cells	Growth inhibition	6.5×10^{-7} to 2.5×10^{-6} (ED50)
Sorenson et al., 1985	Rat alveolar macrophages	Increase in cell volume (membrane effect)	5.0×10^{-5}
Keilova-Rodova, 1949	Fibroblast and osteoblasts of chick embryo	Inhibition of mitosis (binucleate cells)	5×10^{-5} to 10^{-6}
Burger et al., 1988	Tetrahymena pyriformis	Growth inhibition	2.1×10^{-5} (ED_{50})
Sumbu et al., 1983	S. cereviase	Growth inhibition	3.2×10^{-4}
Kubiak and Kosz-Vnenchak, 1983	Chinese hamster cells	Prolongation of mitosis	3.2×10^{-6}
Perlman et al., 1959; Powell, 1966	Rat and mouse fibroblasts	Inhibition of replication	10^{-6}

DNA and Chromosomal Integrity and Mutagenesis

Examination of the effects of patulin on DNA integrity and mutagensis (Table 4) shows that patulin had no effect in such standard tests as the Ames mutagenesis assay for revertants using Salmonella typhimurium (Ames et al., 1973) and the DNA repair test of Williams (1976). For some tests--the "rec" (recombination deficient) assay using Bacillus subtilis, the production of cytoplasmic petite mutants of yeast, and measurement of reversion in a bacteriophage--effect levels ranged from 6.5×10^{-6}M to 1.3×10^{-4}M. However, more consistent results were

Reference	System	Effect or Assay	Effective Dose (M)
Stetina and Votava, 1986	CHO and AWRF cells	DNA strand breaks (single)	6.5×10^{-5}[a]
Umeda et al., 1972	HeLa cells	DNA strand breaks (single and double)	2.08×10^{-4}[a]
Lee and Roschenthaler, 1986	*E. coli*	DNA strand breaks (single and double)	6.5×10^{-5} to 3.2×10^{-4}[a]
Lee and Roschenthaler, 1987	*E. coli* phage (+$CuCl_2$ and NADPH)	DNA strand breaks single double	 2.5×10^{-4} 10^{-3}[a]
Ueno and Kubota, 1976	*B. subtilis* M45 (recombination-deficient)	rec assay (for DNA alteration)	10 and 100 µg/disc[a]
Mayer and Legator, 1969	*S. cerevisiae*, haploid	Cytoplasmic petite mutants	1.3×10^{-4}[a]
Burger et al., 1988	Bacteriophage M13am6H1	Reversion	6.5×10^{-6}[a]
Belitsky et al., 1985	*Drosophila melanogaster* (heterozygous y+/+sn^3 larvae	Somatic mosaic mutation	3.2×10^{-3} (fed)
Lindroth and von Wright, 1978	*E. coli* W3110	polA1 repair assay (for DNA damage)	Negative at 6.5×10^{-5}[a]
Ueno et al., 1978; Wehner, et al., 1978	*S. typhimurium* TA1535, TA1537, TA98 and TA100	His^+ revertant (Ames) assay	Negative at 250 µg/plate[b]
Kuczuk et al., 1978	*S. cerevisiae*, diploid	Mitotic recomb.	Negative at 6.5×10^{-4}[b]
Auffray and Boutibonnes, 1986, 1987	*E. coli* K12 and PQ37	SOS chromotest (for DNA damage)	1 µg/assay and 6.5×10^{-8}[b]
Krivobok et al., 1987	*E. coli* PQ35 and PQ37	SOS chromotest (for DNA damage)	Negative at 1.9×10^{-4}[a]
Mori et al., 1984	Rat and mouse hepatocytes	DNA repair (UDS)	Negative at 6×10^{-5}
Belitsky et al., 1985	Rat and human embryonic liver cells	DNA repair (UDS)	Negative at 1.6×10^{-3}

[a] In the absence of S-9 (metabolic system).
[b] In the absence or presence of S-9.

seen in the measurement of DNA strand breaks *in vitro* in which single- and double-strand breaks were produced at concentrations of patulin as low as 6.5×10^{-5}M for *Escherichia coli* and 2.08×10^{-4}M for HeLa cells. Lee and Roschenthaler (1987) found that the DNA-cleaving activity of patulin was inhibited by superoxide dismutase, catalase, and radical scavengers, suggesting a free radical-mediated process. Strain differences may also be a factor; for example in the SOS chromotest for primary DNA damage or mutagenisis using *E. coli* (Quillardet et al., 1982), both negative and positive effects were reported.

Results are more consistent for measurement of effects on chromosomal integrity (Table 5). Positive results were reported in terms of increase in chromosomal abberations--chromatid but not chromosomal breaks--or in sister chromatid exchange (SCE) for several types of cells, including human peripheral lymphocytes (effect levels, 2.5 to 3.5×10^{-6}M) and fertilized salamander eggs (effect level of 4.1×10^{-4}M). Only for CHV 79 cells and for measurement of SCE were negative results obtained, although chromosomal aberrations were produced in these same

Table 5. Effects of Patulin on *In Vitro* Systems. III. Chromosomal Integrity.

Reference	System	Effect or Assay	Effective Dose (M)
Cooray et al., 1982	Human blood lymphocytes	Sister chromatid exchange	6.5×10^{-7}[a]
Thust et al., 1982	Chinese hamster V79-E cells	Sister chromatid exchange	Negative, 10^{-5}[b]
Kubiak and Kosz-Vnenchak, 1983	CHO cells	Sister chromatid exchange	3.2×10^{-6}[a]
Thust et al., 1982	Chinese hamster V79-E cells	Chromosomal aberrations	2.5×10^{-6}[c]
Withers, 1966	Human leukocytes	Chromosomal aberrations	3.5×10^{-6}[a]
Sentein, 1955	Segmenting salamander eggs	Chromosome fragmentation and inhibition of mitosis	4.1×10^{-4} to 1.6×10^{-3}[a]
Reib, 1975	*Allium cepa* root tips	Inhibition of mitosis and chromosomal aberrations	6.5×10^{-7}[a] Negative for chromosomal aberrations

[a]Measured in the absence of S-9 (metabolic system).
[b]Measured in the absence of presence of S-9.
[c]Effect seen only in the absence of S-9.

cells by patulin at a concentration of 2.5 x 10^{-6}M (Thust et al., 1982). An increase of SCE was produced in human blood lymphocytes at a concentration of 6.5 x 10^{-7}M. Thust et al. (1982) reported no evidence for alkylation of DNA by patulin and thus inferred a direct action of patulin on chromosomal protein to explain its effect on chromosmal integrity. Lee and Roschenthaler (1987) also found no binding of ^{14}C patulin to DNA.

Biochemical Effects

Measurement of biochemical parameters shows that patulin exerted effects--albeit sometimes at high concentrations--on every metabolic system tested (Table 6). Effect levels ranged from 1.6 x 10^{-7}M for inhibition of DNA synthesis to 5 x 10^{-4}M for inhibition of respiration by rat renal explants. Earlier reviews (Singh, 1967; Ciegler, 1977) did not find that patulin affected macromolecular synthesis. Ueno et al. (1969) reported an 80% inhibition of ^{14}C leucine accumulation by rabbit reticulocytes *in vitro* at 3.2 x 10^{-4}M, but the technique did not distinguish protein synthesis from cellular uptake. More recent reports from several laboratories indicated that patulin did indeed affect macromolecular synthesis in several cellular systems and at low concentrations. We have found that the effect of patulin on macromolecular synthesis is probably distinct from, or at least not solely attributable to, its effect on respiration. The effect of patulin on RNA, protein synthesis and respiration of rat liver slices (similar to those for livers from mice, rats, hamsters, and chickens) indicates that RNA and protein syntheses were affected to about the same degree, which is generally what others have found (Schaeffer et al., 1975; Sorenson et al. 1985), but respiration was considerably less sensitive to the toxin (Peters et al., 1977). Hatey and Gaye (1978) presented data demonstrating that the effect of patulin on protein synthesis was on the elongation step of the process, and Moulé and Hatey (1977) indicated that the inhibition of RNA synthesis was due to the effect of patulin on the initiation step. Using "permeabilized" *E. coli* cells, Lee and Roschenthaler (1986) found that DNA synthesis was much more sensitive to inhibition by patulin than RNA or protein synthesis and so postulated that this mycotoxin had selective DNA-damaging activity. DNA synthesis was particularly sensitive in lymphocytes, as shown for those obtained from a human source by Cooray et al. (1982) and for those derived from mice by Robbana-Barnat et al. (1989). The latter workers also demonstrated the higher activity of patulin among seven other potent mycotoxins using mouse lymphocytes.

Table 6. Effects of Patulin on In Vitro Systems. IV. Biochemical (Respiration and Macromolecular Synthesis).

Reference	System	Effect or Assay[a]	Effective Dose or ED_{50} (M)
Braunberg et al., 1982	Rat renal explant	Respiration	5×10^{-4}
Delauney et al., 1955	Polymorphonuclear leukocytes	Respiration	1.5×10^{-4}
Polacco and Sands, 1977	Soybean cultures	Respiration	6.5×10^{-5} (ED_{50})
Gottlieb and Singh, 1964	C. purpurea	Respiration	1.95×10^{-4} (90%)
Sorenson et al., 1985	Rat alveolar macrophages	ATP level protein synthesis RNA synthesis	5×10^{-5} 10^{-6} 10^{-4}
Burger et al., 1988	Tetrahymena	RNA, DNA and protein synthesis	2.1×10^{-5}
Hatey and Gaye, 1978	Reticulocyte, cell free	Protein synthesis (on elongation step)	$3\text{-}6 \times 10^{-4}$ (ED_{50})
Schaeffer et al., 1975	Chang liver cell	Protein and RNA synthesis	1.6×10^{-5}
Peters et al., 1977	Rat liver slice	Protein and RNA synthesis	8.1×10^{-5}
Hatey and Moulé, 1979	Postmitochondrial liver and polysomes	Protein synthesis	8.3×10^{-4}[b]
Moulé and Hatey, 1977	Rat liver nuclei	RNA polymerase and RNA synthesis (initiation)	1.3×10^{-4}
Cooray et al., 1982	Human blood lymphocytes	DNA synthesis	3.2×10^{-7}
Stetina and Votava, 1986	CHO and AWRF cells	DNA synthesis	2.6×10^{-7}
Tashiro et al., 1979	Rat liver	Nuclear RNASE H and RNA polymerase I and II	2.8×10^{-5}
Robbana-Barnat et al., 1989	Hepatoma cells Mouse lymphocytes	DNA and protein synthesis	1.1×10^{-6} 2.4×10^{-7} (both ED_{50})

[a]All processes shown were reduced by treatment at levels shown.
[b]Inhibition ranged from 61 to 83%.

The effects of patulin on activities of some specific enzymes are shown in Table 7. Although several types of enzymes were affected, the effect levels (5.2×10^{-6} to 5.2×10^{-2}M) were generally greater than those for the more complex metabolic systems as shown previously. Enzymes of the terminal electron transport system of Claviceps purpurea were inhibited to a lesser degree than respiration by cell-free extracts

Table 7. Effect of Patulin on In Vitro Systems. V. Biochemical (Enzyme Activity).

Reference	Enzyme	Source	Effective Dose or Dose at % Inhibition[a] (M)
	Terminal Electron Transport Enzymes		
Gottlieb and Singh, 1964	NADH oxidase, Cytochrome C reductase, Cytochrome oxidase, Succinate dehydrogenase, Succinate oxidase	C. purpurea[b] cell-free extracts	(40%) (30%) (30%) (60-80%) (90%) all at 5×10^{-2}
Holscher, 1950	Dehydrogenase (general)[c]	Mouse ascites tumor cells	1.3×10^{-4}
Arafat et al., 1985	Phenylalanyl-tRNA synthetase	Mouse liver	(90%) 10^{-2}
Ashoor and Chu, 1973b	Aldolase	Rabbit muscle	(50%) 1.3×10^{-5}
Ashoor and Chu, 1973a	Alcohol dehydrogenase	Yeast	(50%) 5.0×10^{-5}
Andraud et al., 1965; Reiss, 1979	Urease	?	1.7×10^{-5}
Ashoor and Chu, 1973a	Lactic dehydrogenase	Rabbit muscle	(50%) 6.2×10^{-6}
Karrer and Viscontini, 1947	Co-carboxylase	?	5.2×10^{-2}
Singh, 1967	Glyceraldehyde-3-phosphate dehydrogenase	?	Not inhibited
Phillips and Hayes, 1977	Mg^{2+} ATPase	Mouse brain, liver and kidney	$1.0\text{-}2.0 \times 10^{-3}$

[a]Number in parentheses = % inhibition.
[b]Inhibition of respiration (0_2 consumption of this cell-free extract) was inhibited approximately 60% by 3.2×10^{-4} or growth-inhibitory concentration of patulin.
[c]Substrates = glucose, phenylalanine, succinate, and hypoxanthine.

of this organism, indicating to Singh (1967) that a vulnerable site may occur in the respiratory reactions before the terminal electron chain. Likewise, although aminoacyl-tRNA synthetases were inhibited by patulin (Arafat et al., 1985) the high concentrations required relative to those effective in inhibiting protein synthesis in whole tissue preparations (Table 6) make it unlikely that its mechanism of action on this process can be fully explained by its action on these enzymes. An earlier report by Hoffmann-Ostenhof (1946) that patulin was unable to inhibit the activity of urease could not be confirmed by Andraud et al. (1965) and Reiss (1979) using purified preparations of the enzyme. Finally, Mg^{2+} ATPase in fractions of mouse liver, kidney, and brain were little affected by high concentrations of patulin. In fact, Phillips and Hayes (1977) reported that patulin did not significantly affect Mg^{2+} ATPase at concentrations that affected Na^{+}-K^{+} ATPase in preparations of urinary bladder.

Cell Membranes and Transport

The effects on cell membranes or transport functions of cells are shown in Table 8. Here again the effects of patulin can be seen in many systems and generally at concentrations between 10^{-5} and 10^{-4}M. The effect of patulin in disrupting the membrane and allowing leakage of ions was seen in rat alveolar macrophages and renal epithelial cells in which an enhanced efflux of stored ^{51}Cr and of K ions, respectively, was produced by patulin at concentrations of 5×10^{-5} and 1×10^{-4}M. At concentrations of 5-10 μM, patulin caused a sustained increase in intracellular electronegativity in cultured renal epithelial cells (Riley et al., 1989). Examples are also seen for the effect of patulin on inhibition of ion transport - K^{+} uptake by erythrocytes ($ED_{50} = 10^{-2}$M), active sodium transport in preparations of toad urinary bladder (5×10^{-4}M), and Na^{+}- dependent transport of glycine in rabbit reticulocytes (2×10^{-4}M). In the last effect approximately 40% of the patulin added to the media became bound to the cellular components (Ueno et al., 1976). The enzyme involved in the active transport of Na and K ions across bioelectrically active cell membranes--Na^{+}-K^{+} ATPase (Skou, 1964)--was also inhibited by patulin at levels between 2×10^{-4} and 3.7×10^{-4}M.

Physiological Effects--Isolated Organ Systems

Bridging the gap between in vitro studies using cellular systems and in vivo studies were those in which physiological and biochemical

Table 8. Effects of Patulin on Cell Membranes or Transport-Related Functions.

Reference	System	Effect or Measurement	Effective Dose or ED_{50} (M)
Braunberg et al., 1982	Rat renal explant	Leakage of protein[a]	1.0×10^{-3}
Kahn, 1957	Cold stored human erythrocytes	K ion uptake[b]	(ED_{50}) 10^{-2}
Sorenson, et al., 1986	Rat alveolar macrophages	^{51}Cr release[a]	5×10^{-5}
Phillips and Hayes, 1979	Toad urinary bladder	Electrogenic Na^+ transport[b]	(ED_{50}) 5×10^{-4}
		Na^+-K^+-dependent ATPase[b]	(ED_{50}) 3.2×10^{-4}[c]
Hinton et al., 1989	Renal epithalial cells	K and Na ion flux[a]	(ED_{50}) $1\text{-}2.5 \times 10^{-4}$[d]
Phillips and Hayes, 1977	Mouse brain and liver fractions	Na^+-K^+-dependent ATPase[b]	(ED_{50}) $2.0\text{-}3.7 \times 10^{-4}$
Ueno, et al., 1976	Rabbit reticulocytes	Na^+-dependent glycine uptake[b]	(ED_{50}) 2×10^{-4}
Gottlieb and Singh, 1964	C. purpurea mycelium	Leakage of metabolites	No effect at 3.2×10^{-4}
Singh, 1967	Bovine erythrocytes	Leakage of hemoglobin	No effect
Polacco and Sands, 1977	Soybean cultures	Active phenylanine and threonine uptake[b]	10^{-4}

[a]Process enhanced by treatment.
[b]Process reduced by treatment.
[c]No effect on Mg^{2+} ATPase.
[d]Less effect on Mg^+ ATPase.

measurements were made on isolated organs. At a concentration between 3.25×10^{-4} and 3.25×10^{-3}M, patulin markedly depressed tone and rhythmic activity of the isolated rabbit jejunum or ileum (Schweitzer, 1946). Vick (1959) reported that patulin (1.75×10^{-3} to 1×10^{-2}M) briefly decreased the contraction of the isolated, perfused guinea pig ventricle. Most interesting and somewhat predictive of the findings in later cultured cell studies (Hinton et al., 1989), this mycotoxin as well as a series of other lactones caused the ventricle to lose potassium to the perfusate. Finally, addition of patulin to the medium bathing the isolated guinea pig ileum at final concentrations of 1.95×10^{-5} to 6.49×10^{-5}M inhibited the contractions induced by nicotine and other tested stimulants (Eliasson, 1958).

Table 9. In Vivo Oral Toxicity of Patulin. I. Acute, Subacute LD_{50}.

Reference	Animal	Dosing Regimen	LD_{50} (mg/kg body wt)
Dailey et al., 1977a	Sprague-Dawley rat	Gavage-single dose	32.5
McKinley et al., 1982	Sprague-Dawley weanling rat	Gavage-single dose	55.0
Lovett, 1972	White Leghorn cockerel	Gavage-single dose	170
Hayes et al., 1979	Sprague-Dawley neonatal rat	Gavage-14 day	6.8
McKinley and Carlton, 1980b	Swiss ICR mice (adult)	Gavage-single dose	48.0
McKinley and Carlton, 1980a	Syrian hamster	Gavage-single dose	31.5

IN VIVO EFFECTS

Acute and Subacute Toxicity

In reviewing the in vivo effects of patulin, primary attention was paid to the effects following oral administration of the mycotoxin. The LD_{50} of a single oral dose to adult rats (Table 9) is 32.5 mg/kg body weight to 55 mg/kg body weight; mice and hamsters are as sensitive but chickens are much more resistant. Assuming a noncumulative effect of patulin (McKinley et al., 1982) neonatal rats are extremely susceptible to the lethal effects of this toxin. Some of the specific effects of acute oral administration of patulin to rodents (rats, mice, or hamsters) or adult chickens indicate that whether the administration is in the form of a single dose or multiple doses, a major target site is the upper gastrointestinal tract (Table 10). Effects range from gaseous distention to inflammation and ulceration of the tract. In one study (Dailey et al., 1977a) a dose of 1.5 mg/kg body weight, the maximally tolerated dose for multiple dosing among those used, given five times a week for 20-23 weeks produced severe gaseous distention of the gastrointestinal tract as the only apparent lesion and cause of death. Only 3 hr after oral doses of 9.0 and 18.0 mg/kg body weight to female rats, stomachs were distended, apparently with gas (Friedman, 1989).

Table 10. In Vivo Oral Toxicity of Patulin. II. Acute, Subacute Tissue Effects.

Reference	Animal	Dosing Regimen	Results (Tissue Effects)
Dailey et al., 1977a	Sprague-Dawley rat	Gavage, 1.5 mg/kg, 5x/wk, 20-23 wks	Gaseous distention of GI tract and increased mortality
McKinley et al., 1982	Sprague-Dawley rat	Gavage, 28 and 41 mg/kg, daily, 2 wks or single dose of of 50-65 mg/kg	Inflammation and ulceration of stomach and increased mortality (effect noncumulative)
McKinley and Carlton, 1980a	Syrian hamster	Gavage-single dose of 25-35 mg/kg	Ulceration and inflammation of upper GI tract
		Gavage-16 and 23 mg/kg daily, 2 wks	Alterations same as above; effect noncumulative; cause of death and lesions due to antibiotic action of patulin?
McKinley and Carlton, 1980[b]	Swiss ICR mice	Gavage, 24 and 36 mg/kg daily/2 wks or single dose of 35-45 mg/kg	Ulceration and inflammation of stomach; noncumulative toxicity; death attributed to enterotoxemia
Lembke and Hahn, 1954	Chicks	Gavage-0.2 mg daily for 6 wks	Liver lesions
Lovett, 1972	White Leghorn cockerel	Gavage-66 mg/kg single dose	Hemorrhage of GI tract

Reproductive and Teratology

Few studies have examined the effects of patulin on reproduction by mammals. In an oral study (Dailey et al., 1977*a*) rats were administered the compound by gavage, five times a week for 10-14 weeks daily, before mating, and daily during pregnancy at a dose of 1.5 mg/kg body weight. No maternal toxicity was noted. The principal treatment-related effects were reduction of fetal weights and growth rates of the F_1A and F_2A progeny. There was no significant increase in malformations in fetuses derived from treated dams after 20 days of gestation. In a study (Reddy et al., 1978) in which pregnant mice were

dosed ip on days 5-16 of gestation, the finding was also weight reduction of the fetus at 1.5 mg/kg body weight and absorption of the implanted embryos at 2.0 mg/kg body weight, but no treatment-related teratogenesis. However, indications that patulin may have teratogenic potential were the results with fertilized Leghorn chicken eggs in which doses of 1-2 mg per egg into the air sac produced specific malformations of the limbs--splayed foot and malrotated ankle (Ciegler et al., 1976).

Cytogenesis and Mutagenesis

Measurement of genetic effects after in vivo treatment with patulin gave mixed results similar to those obtained with the in vitro tests. In a test using the host-mediated assay (Gabridge and Legator, 1969) and in which single doses of 10 or 20 mg/kg body weight were adminstered to mice, negative mutagenic activity was obtained with the injected test microorganism, S. typhimurium strain G46. In another test for mutagenesis, the dominant lethal assay (Green et al., 1975) in which male rats were administered patulin, 1.5 mg/kg body weight 5 days a week for 10-11 weeks, negative effects were also noted (Daily et al., 1977a). However, as in the in vitro tests, evidence for an effect of patulin on the integrity of chromosomes was reported. Examination of bone marrow cells of Chinese hamsters, 6 and 24 hr after the last of two oral doses (24 hr apart) of 10 or 20 mg/kg body weight, showed no increase in SCE or change in mitotic index but an increased rate of aberrant mitosis, as evidenced by an increased frequency of chromosomal aberrations. Chromatid and isochromatid breaks as well as chromatid translocations were observed (Korte, 1980; Korte and Ruckert, 1980). These effects were apparently suppressed by giving rats 10% ethanol in place of drinking water for 9 weeks before treatment (Korte et al., 1979).

Biochemical Effects

A few studies which assessed the biochemical effects of orally administered patulin reported a number of interesting findings. In both chicks and rats orally administered patulin diminished the activity of Na^+-K^+-dependent ATPase. In rats the effect was seen in the intestine (Devaraj and Devaraj, 1987) and brain (Devaraj et al., 1982) after a dose of 4 mg/kg body weight was given every other day for 30 days; in the chick the effect was seen both in the kidney and intestine after a dose of 100 mg per bird was administered every 2 days for 30 days (Devaraj et al., 1986a).

In rats treatment of 4 mg patulin/kg body weight every other day for 1 month also produced changes in lipid composition (decrease in total lipids and triglycerides and increase in total cholesterol) in the intestine (Devaraj and Devaraj, 1987), and convulsions--possibly related to the increase in brain acetylcholine and decrease in brain acetylcholinesterase (Devaraj et al., 1982). Fuks-Holmberg (1980) reported no fetal enzyme changes of several enzymes (aminotransferases and dehydrogenases) assayed after gavage-treatment of pregnant rats (3 mg/kg body weight) for days 1-19 of gestation but noted small changes in maternal liver lactic acid dehydrogenase and alanine amino transferase.

Other effects of orally administered patulin in the rats were decreases in levels of blood pancreatic lysosomal markers (cathepsin B and acid phosphatase) and glucose after the feeding of patulin-contaminated diet for 3 months (Devaraj et al., 1986b), and liver and kidney protein synthesis. Protein synthesis, measured as incorporation of a radiolabeled amino acid into the protein fraction of liver and kidney, was depressed 3 hr after a single oral dose of 4.5 - 18.0 mg/kg body weight in a dose-related fashion (Friedman et al., 1989). Glutathione levels in the livers of these treated rats were also decreased but to a lesser extent than was protein synthesis and only in the two higher dose levels of 9 and 18.0 mg/kg body weight, e.g., at 18 mg/kg in male rats protein synthesis was inhibited by 66%, whereas hepatic glutathione level was reduced by only 22% (Friedman, 1989; Friedman et al., 1989). Similarly, at doses between 9 and 18 mg/kg body weight a disaggregation of hepatic polysomes occurred (Friedman et al., 1989), somewhat coinciding with the effect on protein synthesis and indicating that initiation of polypeptide chain synthesis was affected by patulin treatment. Hatey and Moule (1979) also observed inhibition of hepatic protein synthesis and disaggregation of liver polysomes in rats treated with patulin at levels of 1-2 mg/kg body weight, but here treatment was by ip injection. As in the other effects apparently perpetrated by patulin treatment, the question is whether the effect was due to patulin itself or to a metabolite or even a metabolically or nonmetabolically formed conjugate, e.g., glutathione conjugation of some compounds increases their toxicity (Monks and Lau, 1989). Aside from the results with cysteine adducts there is only meager information on this subject. In one study the lethal effect of an ip dose of patulin was increased by pretreatment with a mixed function oxidase (MFO) inhibitor, suggesting that the parent compound is the toxic form of this compound

(Hayes et al., 1979). The findings that chromosomal aberrations of human lymphocytes were produced by patulin *in vitro* only in the absence of an added S-9 metabolic system (Thust et al., 1982) and that chronic treatment of Chinese hamsters with ethanol, a weak MFO-inducer, reduced the rate of bone marrow chromosomal aberrations by patulin (Korte et al., 1979) add weight to this possibility. Related, although perhaps only remotely, is the finding that yeast exposure to patulin results in the apparent induction of enzyme systems rendering the organisms relatively resistant to the growth inhibitory effects of the toxin (Sumbu et al., 1983).

Immunotoxicity

Two experiments carried out in mice indicated that some components of the immune system can be damaged as a result of oral treatment with patulin. In the first of these (Escoula et al., 1988a), in which patulin was administered by gavage at a dose of 10 mg/kg body weight to mice for 4 days, several indices of immune competence were affected. These included a decrease in the number of peripheral lymphocytes (but not neutrophils), a decrease in the absolute number of spleen lymphocytes, particularly the B-cell population with a corresponding increase in the T-cell population, and a decrease in serum immunoglobulin levels. In the second study (10 mg/kg body weight for 4 days) (Escoula et al., 1988b) the immunoglobulin levels were also decreased but resistance to the pathogen *Candida albicans* was enhanced with a corresonding increased neutrophil count, indicating that treatment with patulin compromised only certain components of the rodent immune system. *In vitro* studies by Escoula et al. (1988a) bolstered the case that patulin has an effect on some components of the immune system. For example, at a concentration of 6.5×10^{-6}M patulin caused suppression of the chemiluminescence response of stimulated peritoneal mice and rabbit leukocytes. The mitogenic response of mouse lymphocytes to the mitogens phytohemagglutinin, concanavalin, and pokeweed was reduced by patulin at 1.3×10^{-6}M. A much earlier study (Delaunay et al., 1955) showed that at a concentration of 1.30 to 1.95×10^{-5} patulin inhibited the phagocytic activity of polymorphonuclear leukocytes and macrophages. Thus patulin was able to suppress the cellular and humoral response both *in vitro* and *in vivo*.

Chronic Toxicity

Long-term testing of patulin using oral administration regimens was limited to two studies on rats. In one study (Osswald et al., 1978) weanling female Sprague-Dawley rats (50 per group including a control group) received by oral gavage a dose of 1 mg/kg body weight twice weekly for 4 weeks followed by an oral dose of 2.5 mg/kg body weight twice weekly for an additional 66 weeks. The rats were observed for 110 weeks. There were no differences between control and patulin-treated groups in the incidence of tumor-bearing rats for all tumors or in the incidence, frequency, and latency of individual tumors. There was, however, a low number (statistically not significant) of unusual but benign stomach tumors in the treated group.

Another study (Recci et al., 1981) included groups of weanling male and female FDRL Wistar rats (70 rats per sex per patulin treatment and 100 per sex per control) which were the offspring of rats administered 0, 0.1, 0.5, or 1.5 mg patulin/kg body weight by oral gavage for 4 weeks before mating and during mating, gestation, and lactation. These rats received the same dose levels of patulin, corresponding to the respective parenteral treatment groups by oral gavage three times a week for 24 months. There were no treatment-related tumorigenic effects in terms of total tumors per group, average number of tumors per rat, or tumor latency or differences due to treatment in regard to hematology, clinical chemistry, or urine analytical parameters. Mortality was increased at the high level of the toxin associated with pulmonary and laryngotracheal inflammation.

Another study by Osswald et al. (1978) was carried out with Swiss mice. Twelve pregnant animals were administered twice-daily doses of 2 mg patulin/kg body weight by oral gavage during days 14-19 of pregnancy. The offspring (50 females and 43 males) and dams were observed throughout life. Fewer offspring from patulin-treated than from control rats survived but no treatment-related effect on incidence of tumor-bearing mice was noted. It was reported, however, that malignant tumors of lymphoreticular origin were observed in a high proportion of the surviving dams while a smaller proportion of the dams in the control group developed benign tumors. No statistical analysis or inferences were reported for the latter data.

Protection by Sulfhydryl Compounds

An important aspect of the biological effects of patulin is its apparent reactivity with thiol-bearing compounds. There have been many

examples of how this reactivity can modify and in most cases attenuate the biochemical or toxic effects of the toxin (Table 11). The bacteriostatic and mitostatic activity of patulin can be partially or fully prevented by prior exposure of the toxin to cysteine or other SH-containing compounds. The same is true for most of the SH-dependent enzymes (e.g., aldolase, aminoacyl-tRNA synthetase, lactic acid dehydrogenase, and urease) inhibited by patulin. An exception is yeast alcohol dehydrogenase (Ashoor and Chu, 1973a). The reason for the exception is not known. However, patulin has a much higher affinity for the dehydrogenase than does a similar mycotoxin, penicillic acid, whose inhibition is reversed by the addition of cysteine (Ashoor and Chu, 1973a). As for aldolase (Ashoor and Chu, 1973b), it may be necessary to form the adduct of cysteine and alcohol dehydrogenase by preincubation of both before testing for activity of the enzyme and possible SH-compound protection. Evidence has been presented, however, that once formed, the patulin-cysteine adduct complex is biologically stable, resisting metabolism by strains of E. coli in culture (Lindroth and von Wright, 1978).

Many other processes (Table 11) affected by patulin but markedly less so in the presence of SH-containing compounds have been reported. For example, dermal toxicity of patulin to the mouse or chick embryo was considerably less or absent with the cysteine adduct of patulin and with the glutathione adduct of patulin. The in vitro inhibition of translation, glycine transport, and normal Na and K ion flux by patulin was at least partially reversed by cysteine or glutathione. The in vivo immunosuppression by patulin was partially prevented by cysteine. Patulin acted in an additive manner with another sulfhydryl group-reactive compound, para-chloromercuribenzoate, in inhibiting the activity of aminoacyl-tRNA synthetase. The enzyme-inhibitory action of this compound was also reversed by thiol compounds (DiSabato and Kaplan, 1963; Fondy et al., 1965). However, the protection by sulfhydryl compounds was frequently incomplete (Table 11); in at least one SH enzyme (alcohol dehyrogenase) the action of patulin was not affected by an SH compound, and in one case the effect of patulin (on SCE) was apparently potentiated by cysteine. As indicated in Table 11, simple addition of cysteine to the assay system was sometimes ineffective (preincubation or adduct formation was necessary) as were several thiol compounds other than cysteine (Cavallito and Bailey, 1944).

Singh (1967) reported that glyceraldehyde dehyrogenase, an SH-dependent enzyme, did not react with cysteine and that the reaction

Table 11. Modification of Patulin Activity by Sulfhydryl Compounds[a]

Reference	Compound	Activity	Change
Rondanelli et al., 1957	GSH, Cys, Dimercap	Mitostatic	Prevented
Geiger and Conn, 1945	Cys, Thiogl, Thios (preincubated with patulin)	Bacteriostatic (*E. coli*, *S. aureus*, *B. subtilis*, *S. lutea*) activity	Prevented by Thiogl and Thios; partially by Cys
Ashoor and Chu, 1973b	Patulin-Cys adduct	Inhibition of muscle aldolase	Partial reversal[b]
Arafat et al., 1985	GSH, Mercap, Dithio	Inhibition of mouse aminoacyl-tRNA synthetase	Prevented (and change in UV absorption of patulin)[c]
Arafat et al., 1985	pCMB	Inhibition of aminoacyl-tRNA synthetase	Inhibition by compound additive with patulin
Ashoor and Chu, 1973a	Cys	Inhibition of muscle LDH	Reversed
Reiss, 1979	Cys	Inhibition of urease	Partial reversal
Ashoor and Chu, 1973a	Cys	Inhibition of yeast ADH	No effect
Hatey and Gaye, 1978	GSH	Inhibition of in vitro translation-rabbit reticulocyte cell-free system	Reduced
Hofmann et al., 1971	Patulin-GSH adduct	Dermal toxicity-rabbit skin test	Reduced
Ciegler, 1977	Patulin-cys adduct	Mouse ip toxicity	4 x LD_{50}-no toxicity
Ciegler, 1977	Patulin-cys adduct	Chick embryo toxicity	50 x LD_{50}-no lethality but some teratogenicity
Delaunay et al., 1955	Patulin-cys adduct	Inhibition of respiration-polymorphonuclear leukocytes	Reduced
Ueno et al., 1976	GSH, Dithio	Inhibition of Na^+-dependent-glycine transport-rabbit reticulocytes	Prevented

Table 11 (continued)

Cooray et al., 1982	Cys	Inhibition of DNA synthesis, increase SCE of human lymphocytes	DNA and SCE effect modified[d]
Escoula et al., 1988a	Cys	Immunosuppression	Partially prevented
Hinton et al., 1989	Dithio, GSH	Enhanced Na and K ion flux	Prevented

[a]GSH = glutathione, Cys = cysteine, Dimercap = dimercaptopropanol, Mercap = mercaptoethanol, Dithio = dithiothreitol, pCMB = parachloromercuribenzoate, Thiogl = thioglycolate, Thios = thiosulfate, SCE = sister chromated exchange.
[b]Inhibition not prevented by simple addition of cysteine to assay system; chemical analysis of inactivated enzyme indicated decrease of free -SH and $-NH_2$ groups.
[c]Inhibition partially or totally prevented, depending on specific enzyme.
[d]Cysteine prevented the inhibition of DNA synthesis but potentiated elevation of SCE by patulin.

between patulin and cysteine is slow. The latter is disputed by others who also demonstrated the pH or buffering dependency of the reaction (Hofmann et al., 1971; Ciegler, 1977). Nevertheless, from all the data presented one can generalize that the biological activity of patulin can be modulated by compounds containing free SH groups. In one enzyme studied (aldolase), the diminution of activity by patulin coincides in part with blocking some of the SH (and NH_2) groups (Ashoor and Chu, 1973b), providing compelling evidence that the toxicity of patulin can be at least partially attributed to its reaction with the SH groups of protein. It is logical to surmise, however, that other groups of proteins, particularly the free amino groups, can also be attacked by patulin (Ashoor and Chu, 1973; Ciegler, 1977). That this must be the case is seen in the reports reviewed by Singh (1967) that other non-thiol amino acids protect against the toxicity of patulin. No information has been presented thus far concerning possible adduct formation between patulin or its metabolites and nucleic acids in vivo, although atempts to demonstrate binding of patulin to DNA in vitro have failed (Thust et al., 1982; Lee and Roschenthaler, 1987). Some evidence suggests that patulin, at least in the presence of a bivalent metal ion and a reducing agent, can inflict molecular damage via a free radical mechanism (Lee and Roschenthaler, 1987). Riley et al. (1989) confirm that the excessive blebbing and increase in malonaldehyde formation in renal epithelial cells caused by patulin in vitro can be prevented by treatment with iron chelators. Apparently this treatment delays but does not prevent cell death, suggesting more than one mechanism of toxicity for patulin.

In Vivo Disposition of Patulin

In a study of the disposition of patulin after its ingestion (Dailey et al., 1977b), ^{14}C patulin, 3 mg/kg body weight, was administered by oral gavage to male rats. At various times the animals were sacrificed and tissues, feces, and body fluids were analyzed for radioactive content. Approximately 49% of the radioactivity was excreted in the feces and 36% in the urine; only 2% was expired as $^{14}CO_2$. No unmetabolized patulin was recovered in the urine. The peak of radioactivity in the tissues appeared between 24 and 48 hr. Data from this study were calculated in terms of concentration of patulin equivalents for some of the tissues at 24 and 48 hr (Table 12). The highest amount of radiolabeled material (approximately 3 x 10^{-5}M) was found in red blood cells and appeared to be retained for a number of days after dosing, indicating strong bonding between the red cell membrane and patulin or metabolite(s). A much smaller level was found in the plasma or in the other tissues of the body, although levels in the kidney, liver, or spleen were in the range of in vitro effect levels of patulin (Tables 2-8). The small amount found in the brain, however, would preclude direct action of patulin on this organ as the cause of convulsive and other CNS effects (Devaraj et al., 1982). This is in agreement with the lack of effect of in vivo treatment with patulin on brain Na^+-K^+ ATPase found for other organs (Phillips and Hayes, 1977). Although no unmetabolized patulin was found in the urine, it cannot be extrapolated that the parent compound did not reach some of the tissues at effect levels. The in vivo studies cited show that biologically or biochemically active species of patulin either reached certain target organs (e.g., liver and bone marrow) in effective concentrations or elicited their effects indirectly (e.g., through hormonal mediation).

Interaction

Finally, since patulin is produced by mold species that elaborate other toxic metabolites and are thus likely to be found in some food products concurrently with other mycotoxins (Lillehoj and Ciegler, 1975; Ciegler, 1977), data from studies in which patulin and other mycotoxins were assessed are examined for possible interactions. Dulaney and Jacobsen (1987) found that patulin and either of two other antibiotics (rifampin and bottromycin) have synergistic bacteriostatic activity. Patulin was also synergistic with penicillic acid in regard to toxicity in dogs. Male dogs given sublethal doses of both toxins (penicillic acid, ip, and patulin, iv) presented general toxic signs and pulmonary

Table 12. *In Vivo* Disposition of ^{14}C Patulin Administered Orally to Rats[a].

Organ or Tissue	Concentration of Patulin Equivalent (M) Time After Dosing	
	24 hr	48 hr
Red blood cells	3.16×10^{-5}	3.36×10^{-5}
Whole blood	1.37×10^{-5}	1.59×10^{-5}
Kidney	7.51×10^{-6}	4.32×10^{-6}
Liver	2.48×10^{-6}	2.07×10^{-6}
Spleen	4.47×10^{-6}	10.7×10^{-6}
Lung	1.94×10^{-6}	2.38×10^{-6}
Bladder	1.19×10^{-6}	0.88×10^{-6}
Plasma	1.35×10^{-6}	0.80×10^{-6}
Adrenal	1.18×10^{-6}	1.91×10^{-6}
Ovary	1.16×10^{-6}	0.71×10^{-6}
Testis	0.38×10^{-6}	0.60×10^{-6}
Brain	0.28×10^{-6}	0.33×10^{-6}
Muscle	0.24×10^{-6}	0.20×10^{-6}

[a] Calculated from data of Dailey et al. (1977). Rats administered 3 mg patulin/kg body weight by oral gavage; male and female data combined. (DPM/g tissue or DPM/ml whole blood or plasma converted to concentrations shown by using value given for specific activity of patulin (0.86 μCi/mg).

lesions resembling those in animals given lethal doses of patulin itself (Reddy et al., 1978). In the case of rubratoxin and patulin given ip to male rats, evidence was presented for an antagonistic type of interaction, i.e., patulin reduced the toxic effects of rubratoxin, and rubratoxin prevented the stimulation of hepatic MFO activity by patulin (Kangsadalampai et al., 1981). For 4-day-old chick embryos, however, the toxic effects of patulin and citrinin were simply additive (Ciegler, 1977). Thus, although more work is indicated here, measurement of specific biological systems indicates that interaction between patulin and some other mycotoxins may exist.

SUMMARY

Patulin is moderately to highly toxic to cells and intact organisms after acute or subacute oral administration. It may be considered a moderately potent immunomodulator, effector of chromosomal and membrane damage, and inhibitor of macromolecular synthesis and muscle contraction. Mechanism of toxicity appears to be at least partially via reaction with sulfhydryl and amino groups of proteins and possibly through a process of free radical generation. Little or no evidence has been presented that it is a teratogenic or carcinogenic agent. Additional work is needed in the area of long-term testing and in its in vivo disposition and metabolism.

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Ochratoxin/Citrinin as Nephrotoxins

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INTRODUCTION

The mycotoxins are a diverse group of secondary fungal metabolites. The diversity of chemical structure suggests that toxic mold metabolites may have the potential to cause diseases either after ingestion or contact on the skin. The mycotoxicoses that result from exposure to these compounds may be expressed as dysfunction of nervous system, the liver, the kidneys or potentially many other organs. Clearly, fungal infestation is not a requirement for the production of mycotoxicoses. Although the ability of certain mycotoxins to alter renal function in man has been debated only relatively recently, human contact with fungal toxins is not a new experience. Bagger (1931) had suggested that the earliest encounter of human mycotoxicoses were the ergotism episodes of the Middle Ages. It is likely that earlier occurrences also happened, but undoubtedly the frequency of such occurrences has decreased considerably in modern times. With the development of modern storage techniques for food, fungal contamination, as well as contamination by other microorganisms, has been greatly reduced and often is not considered a serious problem. Indeed, although human mycotoxicoses have not been ignored in recent times, it is nonetheless true that a much greater effort has been expended to address the problem of fungal contamination of animal feeds.

Although animal poisoning with secondary fungal metabolites has been of greater interest in recent times, this does not mean that there are not documented cases of human intoxication. Hayes (1979) summarized several such examples. Epidemiological data have suggested that aflatoxin ingestion by various human populations in several countries have led to severe liver disease as well as hepatocarcinoma. Although some of these studies were not ideal, it was possible in a

general way to demonstrate a cause and effect relationship between the consumption of common food types that were mold-contaminated with measurable quantities of aflatoxins and the resultant disease.

ANIMAL EXPERIMENTS

Experiments with laboratory animals have become quite common in the study of the effects of fungal toxins. Fetocidal effects as well as teratogenesis have been observed with aflatoxin B_1, ochratoxin A, rubratoxin B, T-2 toxins, etc. (Hayes, 1978). Other organ-specific effects also have been reported, for example, rubratoxins (liver), penicillic acid (cardiovascular system), and citreovirdin (nervous system), to cite but a few.

Well documented effects on renal function of certain mycotoxins have also been demonstrated using an animal model. Although morphological changes in the dog kidney have been reported with aflatoxin B_1 and rubratoxin B (Hayes and Williams, 1977) the clearest, best documented effects have been reported with ochratoxin A and citrinin. Some of these latter data will be summarized here, particularly as they contribute to an understanding of the mechanism of action of these compounds. Hence, this overview is not intended to be an exhaustive discussion of animal nephrotoxicity produced by ochratoxin A or citrinin.

Citrinin produces a sustained disruption of renal function in the Sprague-Dawley rat after the administration of a single dose. The renal dysfunction in this laboratory model is characterized as "a high output" renal failure. Daily urine output is three to four times normal with a maximally effective dose of citrinin and urinary osmolality is greatly reduced. Proteinuria also is observed. These effects are accompanied by a rise in blood urea nitrogen (BUN) as well as creatinine, traditional hallmarks for renal failure. Complicated effects also are observed on the excretion of sodium and potassium. Although the effects on the excretion of these two cations is somewhat more variable, the effects observed are clearly not consistent with normal renal function. Morphological studies have demonstrated a disruption of the structure of the convoluted part of the proximal tubules. Distal tubular damage was not observed in the studies. Maximal effects on structure were observed 48-72 hours after administration of a single dose of citrinin with recovery being evident by 96-120 hours. The morphological observations are quite similar to effects noted with other nephrotoxic substances, although the damage

in the rat kidney was confined to the proximal convoluted tubules without evidence of damage to the straight part of the proximal tubule. Details of all of these studies are in the literature (Berndt and Hayes, 1977; Phillips et al., 1980; Lockard et al.,1980). Finally, it should be noted that the overall pattern of response in the rat agreed well with that reported in the literature in other animal species, for example, the pig (Krogh, 1976; Krogh et al., 1973) and poultry (Elling et al., 1975).

Efforts to understand the underlying biochemical events relating to citrinin-induced nephrotoxicity have been less successful. There is little doubt that citrinin must be transported into renal cortical cells in order to exert its toxic effect. If rats are pretreated with probenecid before administration of citrinin, the toxic response is greatly reduced or completely eliminated (Berndt and Hayes, 1982). Furthermore, there is direct evidence that citrinin is transported by the organic anion transporter located in the proximal tubule of the rat kidney (Berndt, 1983). Despite only relatively modest plasma protein binding of citrinin, the above observations suggest if tubular transport of citrinin does not take place, renal toxicity is minimized. Citrinin is metabolized modestly in the rat (perhaps as much as 10-20%), and although the metabolites have not been identified, there is suggestive evidence as to what these metabolites might be (Phillips et al., 1979). Citrinin reduces both hepatic and renal glutathione promptly after administration of a nephrotoxic dose (Berndt et al., 1980). The magnitude of the reduction is not as dramatic as seen with other nephrotoxicants, but this reduction does suggest the possibility of conjugation of citrinin with glutathione. Recovery of organ glutathione content is rapid, consistent with the fact that citrinin's disappearance from the rat is nearly 90% complete within the first 24 hours. The fact that citrinin metabolism does occur suggests that this compound may have the ability to react with certain cellular constituents in addition to glutathione. Studies were undertaken to examine whether or not citrinin might covalently bind to macromolecules in the kidneys (Berndt, 1982). C^{14}-Citrinin was used in these studies and it was found that radioactivity persisted on a TCA-insoluble material after numerous extractions with solvents known to dissolve the parent compound. Although such evidence has been taken with other nephrotoxicants to indicate that covalent binding is the initiating event in the development of nephrotoxicity, it must be noted that other effects of the parent compound have been observed. These effects,

which could be the prelude to the development of citrinin nephrotoxicity, occur very early after exposure of tissues to citrinin, which suggest metabolism may not underlie the toxic event. Berndt and Hayes (1981) found that renal cortical tissue exposed to citrinin in vitro demonstrated a disruption in calcium movement as early as 30-60 seconds after addition of the citrinin to these tissues in an incubation medium. Although it is not clear how disruption of calcium metabolism leads to acute renal failure, this was the earliest documented effect of citrinin, an effect which in all likelihood precedes the metabolic events.

Ochratoxin A also has been studied in laboratory animals. Although the effects of ochratoxin A and citrinin are similar in the Sprague-Dawley rat, their effects on renal function are not identical. Proteinuria and glucosuria have been observed with both substances, as have been reductions in urine osmolality. However, ochratoxin A does not appear to cause the "high output" renal failure, a clear hallmark of the citrinin-induced toxicity in the rat and other species. The differences observed in the nephrotoxic response to these two compounds may relate, in part, to differences in the toxicokinetic characteristics. Jordan et al. (1977) and Berndt et al. (1980) found that repeated, low doses of citrinin failed to produce a nephrotoxic response. On the other hand, repeated dosing with ochratoxin A seemed to be important for the production of nephrotoxicity in the rat (Berndt and Hayes, 1979; Suzuki et al., 1975). Repeated doses of ochratoxin A in the rat did result in a reduction in renal glutathione, an effect similar to that seen with citrinin. Liver glutathione also was reduced, but only late in the treatment protocol at the time when renal dysfunction was observed (Berndt et al., 1980). Ochratoxin A has been observed to produce renal dysfunction in a number of animal species (Huff et al, 1975; Galtier et al., 1981). In this regard citrinin and ochratoxin A show another similarity.

Although many efforts have been undertaken to delineate a mechanism of action of ochratoxin A (Krogh et al., 1974; Meisner and Selanik, 1979; Meisner et al., 1981) most of these studies have only served to focus attention on sites within the kidney where the action may occur. However, Meisner et al. (1983) demonstrated that ochratoxin A decreased mRNA encoding for phosphoenolpyruvate carboxylase in the rat kidney, which suggests a new approach to examine the underlying mechanism of the nephrotoxic response.

Hence, it is clear from the literature that a clear understanding

of the mechanism of toxicity for either citrinin or ochratoxin A is at the present time unknown, as is true with most nephrotoxicants. Based on present observations, it is not even certain that the two toxins have the same mechanism of action. Indeed, further studies may well reveal quite different mechanisms of toxicity for these two nephrotoxins, at least this might be suggested from the apparent differences in the toxicokinetics of citrinin and ochratoxin A.

EFFECTS IN THE HUMAN

Both citrinin and ochratoxin A clearly have effects in a wide variety of animal species. It is not surprising, therefore, that various workers have suggested that one or another of these substances might be involved in the production of nephrotoxicity in the human. In particular, several workers have suggested that the endemic Balkan nephropathy may the end result of such intoxication.

The final answer as to whether or not mycotoxin ingestion causes human renal dysfunction, including endemic Balkan nephropathy, remains to be learned. Barnes (1967) suggested that mycotoxins may be involved in this disease, although other possible etiologic agents also might be involved. Krogh et al. (1977) noted the similarities between porcine nephropathy and the Balkan disease, as well as the presence of ochratoxin A in various foodstuffs and blood samples of affected individuals. However, Barnes et al. (1977) also noted that the most frequent mold contaminant from the same food samples was not an ochratoxin A or citrinin producer. Hence, even at the outset this issue was not clear cut, nor does it remain so today.

Many workers agree that endemic Balkan nephropathy is characterized by more than simply renal dysfunction. Tumors of the urological system occur in humans along with renal dysfunction. Interestingly, renal tumors have been produced in animals treated with ochratoxin A or citrinin. For example, Bendele et al. (1985) demonstrated in a 24-month feeding study that ochratoxin A was a renal carcinogen in B6C3F1 mice and a hepatic carcinogen in female mice of this same strain. Similarly, Aria and Hibino (1983) demonstrated that male, F344 rats fed 0.1% citrinin in an eighty-week study, had a 73% incidence of renal adenomas after forty weeks while the control animals had no tumors. Hence, the two primary mycotoxins thought to be associated with endemic Balkan nephropathy appear to be tumorigenic, consistent with the reports in the human population in the affected areas. However, beyond the superficial evidence of nephrotoxicity and

tumorigenesis, the correlation between exposure to citrinin and ochratoxin A and the occurrence of specific diseases in the human population becomes more difficult to assess. With respect to the tumorigenesis, it is noteworthy that Bendele et al. (1985) failed to demonstrate that ochratoxin A was mutagenic utilizing a battery of bacterial and mammalian cell assays. On the other hand, Kane et al. (1986) did demonstrate that rats fed ochratoxin A for twelve weeks showed evidence of DNA single-strand breaks in both liver and kidney. These observations utilized the alkaline elution method.

After Barnes' initial suggestion that fungal toxins might underlie the endemic Balkan nephropathy, several workers have examined this problem. Krogh and colleagues (Pavlovic et al., 1979 and Krogh et al., 1977) were able to demonstrate the presence of ochratoxin A in foodstuffs. In both studies a variety of cereals and breads were examined in an area of Yugoslavia where the Balkan nephropathy was prevalent. In one study the frequency of occurrence of ochratoxin A contamination of cereal was approximately 9%, but with a considerable variation with some frequencies as high as 43%. These contaminations were higher than found in other areas where the disease was not prevalent. Similar studies were undertaken by Petkova-Bocharova and Castegnaro (1985). These investigators examined cereal samples from an area of Bulgaria where both the Balkan nephropathy and urinary system tumors were well established. Ochratoxin A occurred in approximately 17% of the bean samples in the endemic area as compared to 7% in the control area. The concentrations of ochratoxin A in each of these samples were approximately the same. A similar discrepancy was found in the maize samples tested from the endemic and non-endemic areas. Petkova-Bocharova et al. (1988) also conducted a survey of blood samples taken from subjects who lived in the affected and non-affected areas of Bulgaria. These investigators reported that ochratoxin A positive samples were found more often in the affected area than in the non-affected area. It is data of this type that have led many investigators to suggest that the endemic Balkan nephropathy is caused by certain mycotoxins present in the food and that the affected subjects are exposed on a chronic basis through their normal life styles.

If the hypothesis is true, it would be extremely valuable to conduct controlled, laboratory experiments wherein animals would be treated on an intermittent basis over a long period of time with contaminated food containing ochratoxin A at relatively low

concentrations. This approach would mimic the most likely method of exposure for humans. That is, it is likely that the humans exposed to the fungal toxins received them on an irregular, intermittent basis rather than a fixed dose on continuous day-to-day basis. Such an experiment would not give insights into the mechanism of toxicity, but would help establish whether or not ochratoxin A is a likely etiologic agent. Such an approach also would be consistent with the rather dramatic variability observed in the contamination of foodstuff with ochratoxin A as well as the variability observed in blood samples taken from patients living in affected areas.

Despite the persistence of the suggestions that fungal toxins may underlie Balkan nephropathy, there are dissenters. Hall (1982) has presented an excellent review of these various viewpoints, and these issues continue to be debated. Nichifor et al. (1985) examined the water in affected and non-affected areas. These investigators found that in the endemic areas the water had higher levels of cadmium, chromium, manganese, and cobalt than did the water in the non-endemic areas. These authors believe that ecologic factors such as metal contamination may underlie the disease. Radovanovic (1987) has suggested, based on epidemiological data that the Balkan nephropathy may be caused by a "slow virus" that is transmitted by rodents which contaminate the food and other articles in the households of the affected families. Earlier, Draganescu et al. (1983) found that inoculation of guinea pigs with serum samples from patients with this disease resulted in a morphologically similar disease in the guinea pig. These data show that a blood-borne agent is present in patients with the disease and these authors concluded a role for viruses in the production of endemic Balkan nephropathy.

Attempts to establish clearly that fungal toxins, heavy metals or viruses as causative agents in endemic Bálkan nephropathy have not succeeded. The lack of consistency in finding contamination by any one of these agents in an affected area has continued to be a major problem. In addition, none of the hypotheses have taken into account the possibility that the effects observed are the results of interactions of contaminating agents rather than an effect produced by one or another agent alone. It is extremely likely that if, for example, fungal toxins underlie the disease, individuals who are exposed to one fungal toxin also will be exposed to other fungal toxins. More than one mold has been shown to be a contaminant of various of the cereals studied and these molds can produce a variety

of secondary metabolites. A similar argument could be made if one were to ascribe to the hypothesis that certain metals in the drinking water are responsible for the disease. Of course, it is also possible that an interaction between some of the metals and some of the fungal toxins may underlie the disease. Preliminary data have been obtained which suggest that there is a possibility for fungal toxin interaction (Berndt et al., 1980), but these studies have not been pursued in an effort to truly mimic the long term effects of low-dose administration of the fungal toxins. Although none of these studies offer insight into possible mechanisms by which the endemic Balkan nephropathy occurs, the studies would be useful in delineating which agents are likely to be the causative ones. Mechanistic studies could follow such evidence.

SUMMARY

Although there is evidence of human diseases caused by products of fungal metabolism, it is not certain whether renal dysfunction is such a disease. In animal models it has been demonstrated that ochratoxin A or citrinin are nephrotoxic. These two fungal toxins have similar, but not identical effects in the rat. Citrinin has been found to disrupt renal function in every species in which it has been tested. It has not been suggested as a possible cause of endemic Balkan nephropathy in humans, because it has not been found as a food contaminant in households of affected individuals. Ochratoxin A, however, has been found in plate scrapings and in the blood of those afflicted. The incidents of these observations have been far from 100%, but always higher than in control populations. Other potential causes of the human disease also have been suggested, e.g., viruses, metals, etc. A final solution to the cause of endemic Balkan nephropathy is still uncertain.

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In Vivo and *In Vitro* Toxicity of Cyclopiazonic Acid (CPA)

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INTRODUCTION

There has been considerable interest in recent years concerning the relationships between mycotoxins and diseases of animals and humans. Most of this interest has centered upon the aflatoxins (Jelinek et al., 1989), however, the number of mycotoxins implicated as potential causes of disease has increased steadily. Jelinek et al. (1989) have recently reviewed the worldwide occurrence of mycotoxins in feeds and foods and concluded that expanded efforts to monitor mycotoxin concentrations, including cyclopiazonic acid (CPA), in foodstuffs be initiated. CPA is an indole tetramic acid that was first isolated by Holzapfel (1968). It is produced by Aspergillus and Penicillium species, including A. flavus, A. tamarii, A. oryzae, A. versicolor, P. patulum, P. puberulum, P. veridicatum, P. crustosum and P. camemberti (Dorner et al., 1983; LeBars, 1979; Gallagher et al., 1978; Lansden and Davidson, 1983; Leistner and Pitt, 1977; Luk et al., 1977; Ohmomo et al., 1973; Orth et al., 1977; Rao and Husain, 1985; Still et al., 1978; Trucksess et al., 1987), which have been isolated from various commodities, meats, dried foods and nuts. However, only limited data regarding the natural occurrence of this mycotoxin is available. CPA has been found in the crust of camembert cheese (Still et al., 1978; LeBars et al., 1979). The highest concentration, 4 ppm, was found in a sample stored under unusual (five days at 25 °C) conditions (Still et al., 1978) and most samples contained considerably less CPA (0.1 to 1.5 ppm) (LeBars et al., 1979). The natural

occurrance of CPA in corn was demonstrated by Gallagher et al. (1978) and CPA was found in peanuts at concentrations ranging up to 6,525 ppb (Lansden and Davidson, 1986). In a recent report, Widiastuti et al. (1988) found up to 9 ppm CPA in 21 of 26 Indonesian corn samples.

CPA accumulates in the skeletal muscle of rats (Norred et al., 1985) and, more importantly from a food safety standpoint, chickens (Norred et al., 1988). These findings, together with those given above, suggest that ingestion of CPA by humans is likely, although the extent of exposure is unknown. Interestingly, CPA was produced in culture by A. flavus and A. tamarii isolated from moldy millet associated with kodua poisoning in humans (Rao and Husain, 1985). CPA was also detected in the moldy millet seeds and symptoms consistent with kodua poisoning reportedly were induced in mice by intraperitoneal (ip) injection of millet seed and fungal (isolated from the seed) culture extracts.

Quantitative survey data alone is insufficient for assessment of the potential health hazard posed by a toxin. Toxic effects and the exposures (route of exposure, dosages etc.) required to induce the effects must be determined. Because CPA is produced together with other mycotoxins, including aflatoxin, (Trucksess et al., 1987; Lansden and Davidson, 1986; Widiastuti et al., 1988; Gallagher et al., 1983), the possibility of synergistic or additive toxicities of CPA and other mycotoxins must also be considered. The in vivo and in vitro toxicologic data pertinent to CPA will be reviewed with emphasis on why additional investigations need to be completed before a reasonable toxicologic assessment of CPA can be accomplished.

IN VIVO TOXICITY

CPA toxicity has been studied in several species including rats, mice, guinea pigs, swine, monkeys and chickens. The alimentary tract, liver, kidneys, skeletal muscle and the nervous system are the major target organs of toxicity, although the specific response to CPA exposure differs somewhat from species to species.

Rats

Purchase (1971) first studied the acute toxicity of CPA in rats. The ip and oral LD50s of CPA in males were 2.3 and 36 mg/kg BW, respectively. The oral LD50 in females was 63 mg/kg BW. Hyperesthesia, lethargy, cyanosis and spasms occurred shortly after ip dosing. Lethargy, the principle clinical finding, developed more slowly and spasms were not present prior to death when the rats were dosed orally. The author

speculated that the type and onset of clinical symptoms were related to differences in CPA absorption rates following ip and oral dosing. Purchase (1971) also described the pathogenesis of microscopic lesions found in animals given a single, oral dose of CPA. Degenerative lesions and necrosis were found in the liver, kidney, spleen, pancreas including the islets of Langerhans, salivary glands, myocardium and skeletal muscle of animals given relatively high doses, while nuclear enlargement with peripheral margination of the chromatin was more commonly found in ductal structures of the liver (biliary tree), pancreas (exocrine and endocrine), salivary glands and renal tubules of animals given lower dosages.

Van Rensburg (1984) gave male and female rats (once) weekly oral doses of 12 or 21 mg/kg BW CPA for 14 weeks. Subgroups were periodically killed for histopathologic examinations. Growth rate of high dose males was initially and reversibly decreased; otherwise, no significant clinical toxicity was found. Nuclear changes similar to those found by Purchase (1971) were present in the heart, liver, kidneys, spleen, salivary glands, pancreas, adrenal glands, testes and gastrointestinal tract of high dose animals. Mild cytoplasmic vacuolation (cloudy swelling) and/or focal necroses were also found in the myocardium, liver, kidneys, adrenals and testes. The latter lesions became less severe as the experiment progressed, thus suggesting that metabolic adaption to CPA may have occurred.

Radiolabelled CPA was widely distributed in the tissues of rats following intragastric (ig) (5 mg/kg BW) or ip (1 mg/kg BW) dosing (Norred et al., 1985). The highest specific activities were found in the blood regardless of exposure route, while skeletal muscle, lung, heart, kidney and liver also contained relatively high concentrations of radiolabelled material. CPA and/or CPA metabolites were rapidly eliminated from the body via the feces (presumably through the biliary tract) and to a lesser extent, urine. The biological half-lives for radiolabelled material were approximately 33 and 43 hours following ip and ig dose administration, respectively. These results suggested that the once-weekly dosing regimen implemented by van Rensburg (1984) was not adequate for assessment of the subchronic toxicity of CPA. The findings of Morrissey et al. (1985) further supported this supposition. Morbidity and weight loss were present in rats given 8.0 mg/kg BW CPA perorally on four consecutive days. Mild toxic signs were present in rats given 4.0 mg/kg BW. The liver, spleen and, to a lesser extent, the salivary glands and gastrointestinal tissues of rats given 8.0 mg/kg BW had microscopic lesions attributable to CPA, specifically pyknotic nuclei and focal necroses. Serum alanine

aminotransferase and aspartate aminotransferase activities, but not serum alkaline phosphatase activity, were increased in rats given 8.0 mg/kg BW CPA. Focal degeneration of skeletal muscle has also been found in rats given 8.0 mg/kg BW CPA ig for four days (Norred et al., 1985). Ultrastructural liver lesions, particularly vesiculation of the rough endoplasmic reticulum with ribosome detachment, were found in rats given four daily oral doses of $\geq$ 0.2 mg/kg BW CPA (Hinton et al., 1985).

Morrissey et al. (1987) also studied the the toxicity of low (0.1 mg/kg BW) or high (4.0 mg/kg BW) oral doses of CPA when given to rats alone or in combination with low (0.1 mg/kg BW) or high (2.0 mg/kg BW) doses of aflatoxin B_1. Neither mycotoxin potentiated the effects of the other. CPA alone caused hepatic ultrastructural changes consistent with those reported by Hinton et al. (1985).

The data of Morrissey et al. (1985, 1987), Hinton et al. (1985) and Norred et al. (1985) implied that CPA was toxic when exposure was continuous (daily). To investigate the toxicity of CPA during more prolonged exposures, male rats were given daily oral doses of 0, 0.2, 0.6, 2.0 or 4.0 mg/kg BW CPA for 13 weeks (Voss et al., 1989). Hematologic and serum chemical measurements were made after 7 and 13 weeks. Animals were subjected to necropsy and microscopic tissue examinations after 13 weeks. CPA did not cause overt toxicity at any dose level. Body weight gain, food consumption, clinical appearance, organ weights, hematologic measurements and, with one exception, serum chemical variables of all CPA-exposed groups were similar to the controls. A slight, dose-related increase in serum creatinine was found after both seven and 13 weeks in rats given $\geq$ 2.0 mg/kg BW CPA. The significance of this observation is unclear although the authors speculated that it may have been secondary to mild striated muscle injury not detectable by microscopic examinations or other serum chemical measurements. Mild to focally moderate inflammation of the submucosa of the glandular epithelium of the stomach was found in animals given $\geq$ 0.6 mg/kg BW CPA and was attributed to local tissue irritation resulting from repeated ig CPA administration. Otherwise, no dose-related microscopic lesions were found in any tissue, including the liver, spleen and skeletal muscle. Ultrastructurally, the cisternal arrangement of the rough endoplasmic was disrupted and ribosomal detachment was noted in rats given 4.0 mg/kg BW, but not 2.0 mg/kg BW CPA. These findings were less severe than, but considered consistent with, those found by Hinton et al. (1985).

The absence of overt toxicity in animals given 4.0 mg/kg BW/day CPA

for 13 weeks (Voss et al., 1989) was surprising considering the marked toxicity found in rats given 8.0 mg/kg BW/day and minor effects found in rats given 4.0 mg/kg BW/day CPA for four days by the previous investigators (Morrissey et al., 1985; Hinton et al., 1985). Voss et al. (1989) hypothesized that (a) CPA may elicit a very steep dose-response curve with little or no toxicity occurring below a threshold dose lying between 4.0 and 8.0 mg/kg BW or (b) that there were differences in the CPA preparations used in the different studies. To investigate these possibilities, Voss et al. (1989) gave five male rats 8.0 mg/kg BW CPA for four days according to the experimental protocol described by Morrissey et al. (1985). In contrast to the previous study, no evidence of CPA-related toxicity was found. In a third experiment, five rats each were given ip injections of 0, 4, 9 or 20 mg/kg BW CPA. All of the high dose and one of the rats given 9 mg/kg BW CPA died. These results suggested that the ip LD50 of CPA may be considerably higher than that reported by Purchase (1971). Acute inflammation and necrosis (with abscess formation) of the peritoneal cavity and injection site were present in 3 animals given 9 mg/kg BW (Voss, unpublished observation).

Hill et al. (1986) gave intraperitoneal injections of 0, 0.1, 1 or 5 mg/kg BW/day CPA to male and female rats for 28 days. The highest dose was more than two times the single dose LD50 reported by Purchase (1971). Interestingly, CPA-related mortality was not found at any dose level and clinical signs of toxicity were considered less severe than those reported by Purchase, (1971). Furthermore, the authors reported that the group incidences of CPA-related, microscopic kidney and liver lesions were not a function of dosage, but rather a reflection of individual animals' susceptibility to the effects of CPA. In any event, the findings of Hill et al. (1986) appear more consistent with the ip LD50 data of Voss et al. (1989) than that of Purchase (1971).

Mice

Nishie et al. (1985a, 1985b) studied the toxic and neuropharmacologic effects of CPA in mice and calculated an ip LD50 of about 13 mg/kg BW. Clinical signs included hypokinesia, hypothermia, catalepsy, ptosis, tremor, atypical gait, opisthotonus, dyspnea and prolonged sleep time following barbiturate administration. Altered brain concentrations of dopamine (increase), dihydroxyphenylacetic acid (increase), homovanillic acid (increase), and hydroxyindoleacetic acid (decrease) were also found after CPA administration (Nishie et al., 1985b).

Guinea Pig

Richard et al. (1986) perorally administered CPA to guinea pigs (average weight of 450-500 g) at dosages ranging from 6.25 ug/day to 1.95 mg/day for 30 days. CPA did not cause changes in serum complement activity or cutaneous hypersensitivity to Mycobacterium tuberculosis, even at dosages (1.6 and 1.95 mg/day) which caused hyperesthesia; incoordination; weight loss; increased serum activities of alanine aminotransferase, aspartate aminotransferase, and sorbitol dehydrogenase; increased serum bilirubin; and hepatocellular vacuolation. Microfocal hepatocellular necrosis was found in animals given lesser dosages (0.2 to 0.8 mg/day CPA). Richard et al. (1986) also found lesions in the deep gastric mucosa and submucosa of guinea pigs given 1.95 mg/day CPA, which were similar to those found later in rats by Voss et al. (1989).

The guinea pig has also been a useful model for studying the effects of CPA on skeletal muscle. Skeletal muscle degeneration and necrosis were found in guinea pigs given CPA at dosages of 3.2 mg/kg BW/day for 5 days or 6.4 mg/kg BW/day for 3 days by Peden et al. (1986). Peden (1989) gave 4.0 mg/kg/day CPA to guinea pigs and studied toxicity and the pathogenesis of skeletal muscle lesions over a four-day period using serum chemical, histochemical and ultrastructural techniques. Body weight of the animals progressively decreased following initiation of dosing. Changes indicative of skeletal muscle and/or liver disease such as increased activities of creatine phosphokinase, alanine aminotranferase and aspartate aminotransferase; and increased concentrations of total protein, albumin, urea nitrogen, phosphorus and calcium were found. Myofiber degeneration and necrosis were more prevalent in the gastrocnemius than in other muscles studied. The author attributed this to the relative abundance of fast twitch oxidative-glycolytic muscle fibers, which were the principle fiber type affected by CPA, in the gastrocnemius. Ultrastructurally, CPA caused mitochondrial and sarcoplasmic reticular swellings in affected myocytes. Myofilament condensation, edema and myofibrillar lysis were present in the more severely affected muscles.

Swine

Lomax et al. (1984) recognized that commercially important livestock may be exposed to CPA by ingestion of contaminated corn. They found decreased weight gain, inappetance, reduced activity and bloody diarrhea in pigs given oral doses of 10 mg/kg BW/day CPA. These effects became evident shortly after dosing was begun and persisted until the two-week study was completed. Lesser clinical signs, particularly rough hair coat

and inactivity were also found in pigs given 1.0 mg/kg BW/day CPA. Microscopic lesions, most notably mucosal necrosis and inflammation of the alimentary tract, were found in most pigs given $\geq$1.0 mg/kg/day BW CPA while liver and kidney lesions were confined to pigs given 10 mg/kg BW/day CPA. Hepatic lesions varied from mild cytoplasmic vacuolation to severe, diffuse necrosis. Tubular necrosis and suppurative tubulointerstitial nephritis were the principle renal lesions. Neutrophilic leukocytosis, presumably a response to gastrointestinal necrosis, was found in the high dose group only and was the only hematologic or serum chemical finding.

Canines

Neuhring et al. (1985) gave doses of 0, 0.05, 0.25, 0.5 and 1.0 mg/kg BW CPA twice daily to dogs. The onset of clinical toxic signs was rapid in dogs given the high dose, appearing two to four days after the start of dosing. Symptoms appeared later in those groups given $\geq$0.25 mg/kg BW CPA. All animals given $\geq$0.25 mg/kg BW CPA died or were killed *in extremis*. The remaining dogs were subjected to necropsy and histopathological examinations after 90 days. Degenerative and/or necrotic lesions were found in the vasculature, alimentary tract, liver and kidneys of at least one dog from all groups given $\geq$ 0.25 mg/kg BW CPA. Lesions attributed to CPA and believed to be secondary effects of necrotizing vasculitis were also found in the reproductive organs, skin and brain. Lymphoid depletion and necrosis were also found in the spleen, mesenteric lymph nodes and gut-associated lymphoid tissue.

Primates

Jaskiewicz et al. (1988) studied the effects of CPA, both alone and in combination with aflatoxin B_1, in vervet monkeys. Toxic signs were minimal in animals given 20 mg/kg BW/day CPA perorally for 120 days. Weight gain was comparable to controls and, other than occasional emesis, appearance and behavior of the CPA-exposed monkeys were unremarkable. Histopathologic findings were minor and consisted of nuclear enlargement of the epithelial cells of bile ducts, pancreatic ducts, and renal tubules; tubular casts (kidney); and focal myocardial degeneration. Mild transient increases in the serum levels of alanine and aspartate aminotransferases, lactate dehyrogenase, creatinine, total protein and urea nitrogen were also found and the authors speculated that these changes were related to liver, skeletal muscle or myocardial damage that was insufficient to cause morphologic lesions. Jaskiewicz et al. (1988) also reported the presence of ultrastructural degenerative lesions,

including endothelial thickening, endothelial cytoplasmic vacuolation and separation of the endothelium from the basement membrane of the microvasculature of the heart and kidneys. Other ultrastructural lesions were found in the myocardium (myofibrillar lysis, mitochondrial and lysosomal accumulation) and liver (dilation of the rough endoplasmic reticulum). It is easy to speculate that the ultrastructural vascular lesions represented a less severe form or modification of the vasculitis found in CPA-treated dogs by Neuhring et al. (1986).

In a second experiment, Jaskiewicz et al. (1988) did not find evidence for synergistic effects when CPA and aflatoxin B_1 were simultaneously administered to vervet monkeys. These results were in agreement with those of Morrissey et al. (1987) as discussed above.

Sheep

Cole et al. (1988) reported that milk production of ewes given 5 mg/kg BW/day CPA on two days decreased significantly within 24 hours of the first dose. Milk production increased to pretest levels following cessation of exposure.

Chickens

The chicken is of particular importance because of its utilization as a meat source by humans and its associated commercial importance. The latter point was dramatically illustrated by an outbreak of Turkey "X" disease in England in which 100,000 poults died (Blount, 1961). Because some of the poults developed neuromuscular symptoms, most notably opisthotonus, consistent with CPA intoxication, Cole (1986) retrospectively suggested that CPA, along with aflatoxin, may have contributed to the disease outbreak.

Wilson and Hagler (1989) studied the LD50 of CPA in day-old male and female broiler chicks, male and female turkey poults, unsexed ducklings and sexually mature quail. The oral LD50 in chicks and poults was comparable, ranging from 12.0 mg/kg BW in male chicks to 19.0 mg/kg BW in male poults. Sex differences were not apparent. In ducklings, the LD50 was significantly higher, approximately 38 mg/kg BW. The LD50 in male quail was 69.6 mg/kg BW. The LD50 in female quail could not be calculated precisely, although the data suggest it was between 25 and 50 mg/kg BW. In a separate experiment, Wilson and Hagler (1989) found that aflatoxin B_1 did not significantly enhance the acute toxicity of CPA to chicks, nor did CPA enhance aflatoxin B_1 toxicity.

Cole et al. (1983) fed chickens diets experimentally contaminated with 10, 50 or 100 ppm CPA for seven weeks. Feed conversion was significantly reduced in birds fed the 100 ppm diets. Cumulative mortality in high dose birds was approximately 60 percent, while survival was unaffected by diets containing $\leq$ 50 ppm CPA. Inflammation of the proventriculus and crop was found in chickens fed the 50 and 100 ppm diets. The lesions were generally more severe at the highest dosage, with necrosis and ulceration being common. Hepatic lesions (hepatocellular vacuolation, necrosis, chronic hepatitis) splenic necrosis and myocardial/epicardial inflammation were found in birds fed diets containing 100 ppm CPA and, to a lesser extent, in birds fed 50 ppm CPA. Bile duct proliferation was found in approximately 30 percent of the chickens fed the high dose diet. Chronic hepatitis was the only noteworthy lesion found in birds fed the 10 ppm diets; the group incidence was about 30 percent.

Cullen et al. (1988) studied the subchronic effects to broiler chicks of daily oral exposure to 0, 1, 2 or 4 mg/kg BW/day CPA for 23 days. Proventricular necrosis and hyperplasia with mucosal and submucosal inflammation were found in birds given the two highest dosages. Hepatocellular vacuolation was found in most high dose birds. Skeletal muscle degeneration characterized by edema, hyalinization and fragmentation of myofibers, macrophage aggregation and satellite cell proliferation was noted in approximately one-third of the high dose birds, but not in birds given 2 mg/kg BW/day CPA. Plasma creatine phosphokinase activity was increased in association with the muscular lesions. Interestingly, no myocardial lesions attributable to CPA were found. Focal coagulative necrosis of the spleen was in found approximately one-third of the birds given 4 mg/kg BW and vacuolation of the exocrine pancreas was present in one-third of the birds given $\geq$ 2 mg/kg BW CPA, respectively. Although these lesions were consistent with those found by Dorner et al. (1983), they occurred in birds given daily dosages approximately ten times less, as calculated by Cullen et al.(1988), than the high dose given in the latter investigation.

CPA accummulated in the muscle of chickens (Norred et al., 1988) given 0.5, 5.0 or 10.0 mg/kg BW orally. Greatest skeletal muscle concentrations were found within 3 hours after dosing, regardless of dosage. CPA was rapidly eliminated from the muscle thereafter, although the rate of elimination was slower in those birds given 10 mg/kg BW compared to the other groups. The authors found a correlation between toxicity and the rate of CPA elimination from muscle of individual birds

given 10 mg/kg BW CPA, but not at the lower dosages. Cole et al. (1988) gave daily doses of 0, 1.25, 2.5, 5.0 or 10 mg/kg BW/day CPA to laying hens. Eggs with cracked and/or thinned shells were laid by all hens given $\geq$ 5.0 mg/kg BW/day and egg production ceased in these groups within 3 days after the first dose administration. All hens given 5.0 or 10 mg/kg BW/day CPA died within seven days. Egg production was severely reduced (about 25 percent) in hens given 2.5 mg/kg BW/day.

Porter et al. (1988) found alterations in brain neurotransmitor/neurometabolite concentrations 96 hours following peroral administration of 0.5, 5.0 or 10.0 mg/kg BW CPA to four week old chickens.

IN VITRO STUDIES

In vitro studies of CPA are extremely limited. CPA was not mutagenic, either with or without the S9 fraction, when tested by Wehner et al. (1978) using the *Salmonella typhimureum* his- reversion assay of Ames (Ames et al., 1975). In contrast, Sorenson et al. (1984) reported that CPA was mutagenic to *S. typhimureum* strains TA98 and TA100 if the S-9 fraction was present. Sorenson et al. (1983) attributed the difference in results between the two studies to the higher concentration (1.0 micromoles/plate versus 0.75 micromoles/plate) of CPA used in their investigation.

The most extensive *in vitro* investigations of the cytologic effects of CPA have focused upon the interactions between CPA, biomembranes and the calcium transport process; important factors for cellular function in general and muscle cell function in particular. Riley et al. (1985) demonstrated that CPA potentiated the accummulation of tetraphenylphosphonium (TPP^+) in renal epithelial cells (LLC-PK) *in vitro*. Further investigation (Riley et al., 1986) showed that CPA-potentiated TPP^+ accumulation was linear, nonsaturable and inhibited by a number of factors including oubain, high-potassium medium, n-ethylmaleimide, dinitrophenol, tetrahexylammonium and, most effectively, carbonylcyanide-m-cholorophenylhydrazone. TPP^+ was not, however, in passive equilibrium but was found tightly bound to the plasma membrane and mitochondria.

Riley et al. (1987) also demonstrated that CPA-potentiated TPP^+ uptake occurred in proliferating rat skeletal muscle myoblasts (L6), although TPP^+ uptake decreased as the cells aged. CPA did not potentiate uptake by nonproliferating rat hepatocytes. Furthermore, TPP^+ uptake was potentiated by CPA in three cell types, renal (LLC-PK), muscle (L6) and primary rat hepatocytes, permeabilized by freeze-thaw lysis (Riley et al.,

1987). Based upon the results of these investigation it can be concluded that CPA induces an electrical alteration on the cytoplasmic surface of the plasma membrane (Riley, personal communication). CPA had little or no effect on Na^+ and K^+ flux or content in renal cells (Riley et al., 1987; Riley et al., 1989).

CPA was subsequently shown (Goeger et al., 1988) to inhibit calcium uptake and calcium-dependent ATPase in sarcoplasmic reticulum vesicles and to alter Ca^{++} flux in L6 muscle cells (Riley et al., 1989). CPA also inhibited calcium binding to a high affinity binding site on the sarcoplasmic reticulum vesicles and phosphorylation of the sarcoplasmic reticulum vesicles (Goeger and Riley, 1989). Interestingly, CPA imine also inhibits calcium transport and ATPase activity of sarcoplasmic reticulum vesicles while tenuazonic acid, a non-indole tetramic acid, is not inhibitory (Riley and Goeger, 1989). This suggests that the indole nucleus plays an important role in CPA-membrane interactions.

CONCLUSION

There is insufficient evidence to conclude whether or not CPA poses a significant health risk to animals or humans. The available evidence suggests that the likelihood of CPA exposure is high although the frequency and level of exposure is unknown. Reports of the natural occurrance of CPA in feeds and foodstuffs are confined to one study of peanuts (Lansden and Davidson, 1983), two studies of corn (Gallagher et al., 1978; Widiastuti et al., 1987), one study of millet (Rao and Husain, 1985) and studies of cheese (LeBars et al., 1979; Still et al., 1978). Therefore, additional investigations to determine the concentration of CPA in feeds and foods, including meat, milk and eggs, must be completed before reliable estimation of exposure can be made.

Secondly, the available toxicity data are contradictory. Hill et al. (1986) first proposed that strain and age differences of the rats used in their studies versus those used by Purchase (1971) could account for the greater toxicity observed by the latter investigator. The failure of Voss et al. (1989) to reproduce the findings of Morrissey et al. (1985) and Hinton et al. (1985) using the same experimental protocol, including strain and sex of the rats, strongly suggests that other factors apparently influence the toxicity of this compound. Because the CPA used by Voss et al. (1989) was isolated from P. griseofulvum and that used in the latter investigations was reportedly isolated from A. flavus, it is reasonable to believe that trace contaminants present in the mycotoxin preparations may have qualitatively and quantitively differed. CPA is a

chelator of divalent cations (Gallagher et al., 1978; Holzapfel, 1968) and therefore, the possibility that CPA toxicity may be modified by the particular combination of cations present in the dosing medium, diet or mycotoxin preparation should be investigated. Secondly, the naturally occurring form of CPA, alpha-CPA, may undergo epimerization to iso alpha-CPA under certain basic conditions (Kozikowski et al., 1985). Interestingly, Clements (personal communication) found two peaks, both of which had mass spectra consistent with CPA, during capillary gas-liquid chromatographic/mass spetral analysis of a commercial CPA standard. The epimeric configuration of CPA in the dosing medium has not been reported for any study completed to date. Beause of the relatively limited solubility of CPA in aqueous media (Purchase, 1971) 1 N sodium bicarbonate solution was selected for use as the dosing vehicle in the (rat) toxicity investigations of Purchase (1971), and later by other investigators including Van Rensburg (1984), Hill et al. (1986), Morrissey et al. (1985), Hinton et al. (1985) and Voss et al. (1989). In contrast, studies using guinea pigs (Richard et al., 1986; Peden et al., 1985), dogs (Neuhring et al., 1986) and swine (Lomax et al., 1984) have utilized gelatin capsules for intragastric dosing of CPA without the use of basic solutions. Because toxicity was apparent in all of these species at relatively low dose levels ($\leq$ 10 mg/kg BW), Voss et al. (1989) suggested that future rodent studies be done by, preferentially, mixing CPA directly in the feed or other means avoiding the use of alkaline solutions. It would also be interesting, in view of the apparently contradictory results of rat studies, to compare the results of additional swine and/or dog studies using the same experimental protocol, but a different batch of CPA. Wilson and Hagler (1989) found no differences in the LD50 of CPA dissolved in corn oil and CPA dissolved in 1 N sodium bicarbonate. Cullen et al. (1988) calculated the daily exposures (mg/kg BW) incurred in chickens fed CPA fortified diets by Dorner et al. (1983) and compared them to the oral doses (gavage using corn oil as the vehicle) given in his investigation. He concluded that CPA induced toxicity in chickens at considerably lower doses when given by gavage than when given by dietary admixture, most likely due to differences in absorption from the gut. Mortality data reported by Cole et al. (1988) for laying hens given daily doses of $\geq$5 mg/kg BW/day CPA (given in gelatin capsules) suggests that the mycotoxin is more potent when administered without the use of a vehicle, although physiologic and metabolic differences between laying hens and broilers may have modified the effects of CPA. However, given the apparently contradictory data obtained from rodent studies, it

is possible that there were chemical differences in the batches of CPA used in these investigations.

Thus, it is apparent that before we can accurately determine the dosages of CPA which induce adverse effects in birds and mammals, explanations for these apparent discrepancies must be found. The stereochemical configuration(s) of CPA in dosing media used for toxicity studies and in naturally contaminated commodities, interactions between CPA and other dietary components (particularly metals) and , if warranted, the impact of these variables upon CPA absorption, tissue distribution and toxicity should be determined. These data, along with more detailed surveys of naturally occurring CPA concentrations in commodities and foods will allow for reasonable assessment of CPA toxicity and its potential risk to animal and human health.

SUMMARY

CPA is produced by a variety of Aspergillus and Penicillium species which have been isolated from a foodstuffs including cured meats, dried beans, corn, peanuts and cheese. CPA accumulates in skeletal muscle of rats and, more importantly from a food safety aspect, chickens following oral dosing. Reports of the natural occurrence of CPA are limited to peanuts (one report), corn (two reports), millet (one report) and cheese (two reports). Although target organs vary somewhat from species to species, degenerative, necrotic and/or inflammatory lesions of the liver, kidneys, spleen, alimentary tract, lymphoid tissue, skeletal muscle, and/or myocardium are typically found in rats, chickens, dogs, guinea pigs, swine and vervet monkeys subsequent to exposure. Atypical nuclei may be found the ductal structures of the pancreas, salivary glands and biliary tree after CPA exposure has occurred, especially in rats and monkeys. Weight loss and neurologic/neuromuscular symptoms are typical clinical findings in intoxicated animals. Available data suggests that CPA dosages required to elicit toxic effects in rats and chickens vary considerably from study to study. These differences may be attributable, at least in part, to differences in dosing vehicles and experimental protocols used. However, new information suggests that these differences may also reflect chemical differences in the batches of CPA used for the experiments. Therefore, before an assessment of the importance of CPA as a natural contaminant of feeds and food can be made, additional studies are needed to further document the natural occurrance of CPA, to determine the reason for the apparent differences in the potency of CPA batches used in the various studies, to determine experimental conditions that may modify

CPA toxicity in vivo and to reevaluate the potential acute, subchronic and chronic toxicity of CPA using dosing methods that are as close as possible to natural exposure conditions.

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Immunotoxicity of Trichothecene Mycotoxins

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INTRODUCTION

The trichothecene mycotoxins are sesquiterpene alcohols or esters. Several genera of imperfect fungi, including; Trichoderma, Trichothecium, Myrothecium, Stachybotrys, Cephalosporium, and Verticimonosporium are known to produce trichothecene mycotoxins, most notable, however, are several Fusarium species. To date approximately 100 naturally occurring trichothecene mycotoxins have been identified. However, the rarity of most has limited toxicology research, primarily, to the study of; 4-deoxynivalenol (DON, vomitoxin), 3-acetyl-deoxynivalenol (3-acetyl-DON), diacetoxyscirpenol (DAS, anguidine), fusarenon-X (FUS-X), nivalenol (NIV), and T-2 toxin (T-2). Often described as secondary, plant metabolites, without obvious benefit to the organism, mycotoxins are believed to be produced in response to various environmental stresses. Trichothecene-producing fungi are distributed widely, and found in temperate as well as subtropical climates.

Both human and animal toxicoses, resulting from ingestion of contaminated grains or grain products, have been described (reviewed in Rodricks et al., 1977; Wyllie and Morehouse, 1978; and Ueno, 1983). The most devastating human intoxications occurred in the U.S.S.R. during the period 1942-1947 (Mayer, 1953), though recently, Bhat et al. (1989) reported on a possible intoxication of people inhabiting the Kashmir Valley, India. The authors found evidence of trichothecene mycotoxin contaminated grain products. Several countries have established exposure limits, ranging, for DON, from 500-2000 µg/kg in products for human consumption, to 4000 µg/kg for animal feed ingredients (Jelinek et al., 1989). Recently, Fusarium oxysporum has been isolated from immunosuppressed patients (Anaissie et al., 1986; Strum et al., 1989). In Doyle and Bradner's (1980) review of trichothecene, mycotoxins the

authors listed F. oxysporum as a producer of neosolaniol. However, whether or not the sequellae of F. oxysporum infection includes the production of this trichothecene mycotoxin remains to be established.

The trichothecenes are perhaps best recognized for their potent inhibition of both DNA and protein syntheses. Reported IC_{50} values (the concentration resulting in a 50% inhibition) for the inhibition of DNA synthesis in human, mouse, or rat mitogen-stimulated lymphocytes are presented in Table 1. Of the tested trichothecenes, T-2 is the most potent inhibitor of DNA synthesis. Various aspects of trichothecene mycotoxin inhibition of protein synthesis have been reported (Ueno et al., 1973; Liao et al., 1976; and McLaughlin et al., 1977). Melmed et al. (1985) reported an IC_{50} value of 0.8 ng/ml for T-2 inhibition of protein synthesis in the cultured macrophage cell line, J774. In similar experiments, the authors reported IC_{50} values for puromycin and cycloheximide to be 10 µg/ml and 40 ng/ml, respectively. Results of a comprehensive structure-activity study of the cytotoxicities of various analogues, and reaction products derived from T-2, indicated the most potent to be T-2 and DAS (Anderson et al., 1989). Anguidine has undergone clinical trials in the US as an anticancer agent (Goodwin et al., 1978; Yap et al., 1979), its success, however, has not been remarkable. Various structural modifications of Anguidine have, however, resulted in increased tumor cell cytotoxicity (Kaneko et al., 1982).

DISCUSSION

Though the impetus for this review is discussion of trichothecene mycotoxin associated immunotoxicity, it should be noted that these mycotoxins are not without systemic effects, as such, many of the trichothecenes have multifaceted toxicologies, which have been described in detail (Ueno, 1983; Beasley, 1989; Wyllie and Morehouse, 1978). The effects of trichothecene mycotoxins on the immune system of animals have recently been reviewed in detail (Taylor et al., 1989a). It is clear that some of the trichothecene mycotoxins suppress immune function. The following discussion has been divided into four sections. First, a brief orientation to the immune system; second, several aspects of trichothecene mycotoxin-associated immunodysfunction are discussed. In summary, a working paradigm for the immunodysfunction is suggested and discussed, and in conclusion, the potential risks to both human and animal health are discussed.

Table 1. IC_{50} Values for the Inhibition of DNA Synthesis in Mitogen-Stimulated Lymphocytes.

COMPOUND	CELL	IC_{50} (ng/ml)	REFERENCE
T-2	Human, PBL	0.5-2.5	Forsell et al.,1985
T-2	Human, PBL	1.5	Cooray, 1984
T-2	Mouse, Splenocyte	<0.25	Rosenstein and Lafarge-Frayssinet, 1983
DON	Human, PBL	220	Atkinson and Miller, 1984
DON	Rat, Splenocyte	90	Atkinson and Miller, 1984
3-Acetyl-DON	Human, PBL	1060	Atkinson and Miller, 1984
3-Acetyl-DON	Rat, Splenocyte	450	Atkinson and Miller, 1984
3-Acetyl-DON	Human, PBL	100-200	Tomar et al., 1986
3-Acetyl-DON	Mouse, Splenocytes	150-200	Tomar et al., 1987
FUS-X	Mouse, Splenocytes	500	Masuda et al., 1982

Table 2. Summary of Trichothecene Mycotoxin Effects on Host Resistance in Mice.

TOXIN	STRAIN,SEX	DOSE,DURATION	INFECTING ORGANISM	MORTALITY	REFERENCE
T-2	Swiss, m	10-20 ppm, diet 2-3 weeks	Herpes Simplex Virus type I, ip	Increased	Friend et al., 1983
T-2	ddy, m	0.1 mg, po, days 8,10,...,18 after infection	Mycobacterium bovis,iv	Increased	Kanai and Kondo, 1984
T-2	C3H/HeN, f	1.0 mg/kg, po single injection 24 hr before infection	Salmonella typhimurium,po	Increased	Tai and Pestka, 1988a
T-2	ICR, f	4 mg/kg, po same day as infection	Listeria monocytogenes,ip	Increased	Corrier et al., 1987
T-2	ICR, f	4 mg/kg, po days 4 or 2	Listeria monocytogenes,ip before infection	Decreased	Corrier et al., 1987
DAS	Swiss, m	1.12 mg/kg, ip days 3-9 after infection	Candidia albicans,iv	Increased	Frometin et al., 1980
DAS	ICR, f	3.0 mg/kg, po, days 2 and 1 before infection	Listeria monocytogenes,ip	Increased	Ziprin and Corrier,1987

Immune System

Structurally, the immune system has both nonlymphoid cellular, and primary and secondary lymphoid tissues. The nonlymphoid cellular components include the various granulocytic leukocytes and the mononuclear macrophages. In mammals, the principal postembryonic origin of these immunocytes is bone marrow. The major classes of the leukocytes, both myeloid and lymphoid, arise from distinct progenitor cells. Following differentiation and maturation, the myeloid cells become granulocytes and monocytes. The granulocytes are classified as either neutrophils, eosinophils, or basophils, while the monocytes become macrophages after leaving blood and entering tissues. The lymphoid cellular components include both B and T cells. In mammals, mature B cells arise from the bone marrow. Although T-cell precursors arise in bone marrow, T cells mature in the thymus. Both the bone marrow and thymus are primary lymphoid tissues. Upon immigration (maturation) from primary lymphoid tissues, both B and T cells may take up residence in secondary lymphoid tissues or recirculate in blood and/or lymph. Secondary lymphoid tissues include spleen, tonsils, lymph nodes, and Peyer's patches of the ileum.

Functionally, the components of the immune system are involved in many processes such as tissue repair, the confinement, removal, or killing of infectious organisms, and recognition and killing of transformed cells. Additionally, the immune system may integrate with both the nervous and endocrine systems. The B cells, following antigenic activation, are ultimately transformed into antibody secreting plasma cells. The T cells fill several capacities; they are recognized as either regulators, including helper and suppressor-type T cells, or effectors, including cytotoxic and delayed-type hypersensitivity T cells. Most B cells depend upon T cells and their products in their transition to antibody-secreting plasma cells. The requirement for T-cell participation is antigen dependent; therefore, antigens and the B-cell responses which they elicit, have been categorized as either T-dependent or T-independent, based upon their T-cell requirement. Thus, B cells may be stimulated by either T-dependent or T-independent mechanisms. Two principal phagocytic cells in mammals are the macrophage and neutrophil. Macrophages are the primary, antigen presenting cells to both T- and B-cells, and are therefore early participants in both T- and B-cell responses.

Trichothecene Mycotoxin Immunotoxicity

Although the effects of trichothecene mycotoxins on macrophage

function, antibody response, resistance to infectious organisms, graft rejection, delayed-type hypersensitivity, and mitogen-induced lymphoproliferation have been studied, emphasis herein has been given to the first three topics. For a more indepth discussion of trichothecene mycotoxin-associated immunotoxicology the reader is referred to recent reviews (Taylor et al., 1989a; Otokawa, 1983).

Host Resistance. The biologic mechanisms of host resistance depend to a large degree on the nature of the infecting organism. The macrophage is often a primary component of the host resistance mechanism as these cells may serve to recognize, contain, and degrade, as well as present antigens to either T or B cell. Subsequently, T cells may mount a response toward the infecting organism giving rise to cytolytic-T cells, and B cells may produce antibodies toward infectious organisms which serve to opsonize the pathogen, thus enhancing in its removal. Summarized in Table 2 are some of the known effects of trichothecene mycotoxins on host resistance to infectious organisms. Resistance to fungal (Candidia albicans), bacterial (Mycobacterium bovis, Listeria monocytogenes, Salmonella monocytogenes), and viral (Herpes Simplex Virus, type I) infections have been compromised by exposure to either T-2 or DAS. Most often mortality, a common and ultimate endpoint in the analysis of host resistance, is increased by exposure to either T-2 or DAS at the time of infection, or when exposure to a toxin is continued after infection. However, an interesting phenomenon was observed when mice were treated with T-2 prior to L. monocytogenes infection, that is, mortality decreased in this model (Corrier et al., 1987). In a similar study of DAS, no protection against L. monocytogenes was noted (Ziprin and Corrier, 1987). The enhanced resistance to L. monocytogenes infection which occurred when T-2 was administered prior to infection is one of several observations which suggest that T-2 has immunomodulatory activity. At present, no tenable hypothesis has been provided to explain the observed protection against L. monocytogenes which is afforded by T-2 pretreatment. Listeria monocytogenes is a gram-positive, facultative, intracellular bacteria. While resistance to L. monocytogenes infection involves both macrophages and T cells, it has been demonstrated recently that tumor necrosis factor alpha (TNF-α), a polypeptide product of macrophages, may be an important mediator of resistance against L. monocytogenes infection (Nakane et al., 1988; Desiderio et al., 1989). The authors reported that resistance to L. monocytogenes was greatly reduced if the animals were treated with an antibody to TNF-α. It is at least plausible that macrophage activity

is stimulated by exposure to T-2, resulting in increased production of TNF-α, which when increased prior to L. monocytogenes infection affords protection. Preliminary results in our laboratory indicate that concomitant T-2 and endotoxin treatment results in a much greater serum level of TNF-α in endotoxin treated mice. This may be an important observation in understanding how T-2 treatment results in its immunomodulation of resistance to L. monocytogenes.

Antibody Response. Antibody response following in vivo exposure to various tricothecenes has been studied and summarized in Table 3. Antibody response in trichothecene mycotoxin treated animals has been studied using both T-dependent and T-independent antigens, and an interesting dichotomy has been observed. It appears that within effective dose ranges, T-independent responses increase while T-dependent responses decrease as a result of exposure to either T-2 or DAS. An increased T-independent response has also been reported for 3-acetyl-DON. As discussed earlier, the T-dependent and T-independent responses differ in their respective T cell dependency.

Decreased thymus weights have often been observed following treatment with T-2 toxin. In vivo, the thymus, a primary lymphatic tissue, is considered to be the principal reservoir of mature T cells. The effect of T-2 toxin on the thymus, whether direct or indirect, may be a mitigating factor accounting for altered antibody responses in toxin treated animals. We have observed that after a 2-week treatment with 2.5 mg/kg body weight T-2, the percentage of splenic T cells is reduced markedly (50%), and attributable primarily to a decreased T-helper cell population (unpublished). The increased T-independent responses which have been observed following treatment with either T-2, DAS, or 3-acetyl-DON may also be due, in part, to a change in the T-cell pool. It has been suggested that a hormonal imbalance may be associated with the effects of T-2 on antibody response, as the principal glucocorticoid hormones of swine and mice, cortisol and corticosterone, respectively, increase following treatment with T-2 (Rafai and Tuboly, 1982; Taylor et al., 1989b). Both the immunosuppressive and the lympholytic effects of these hormones are appreciated . Additionally, the production of cytokines which aid in the development of the antibody response may be affected.

The release of interleukin 1 (IL-1), has been shown to increase following in vitro exposure of peritoneal macrophages to T-2 toxin (Miller and Atkinson, 1986). IL-1 has multiple targets in addition to being required for the initial activation of T cells. Efrat et al.

Table 3. Summary of Trichothecene Mycotoxin Effects on Antibody Response in Mice.

TOXIN	STRAIN,SEX	DOSE,DURATION	ANTIGEN	RESPONSE	REFERENCE
T-2	Balb/c, m	0.5-2 mg/kg ip, days 1-3 before, and days 4-7 after antigen	TI[a,b]	Increased	Rosenstein et al., 1981
T-2	CD-1, m	2.5 mg/kg, po days 1,4,6,8 before, and days 1 and 3after antigen	TI[a]	Increased	Taylor et al., 1989c
T-2	Swiss, m	0.5-2 mg/k ip, days 1-3 before, and days 4-7 after antigen	TD[c]	Decreased	Rosenstein et al., 1979
T-2	CD-1, m	$\geq$ 2.5 ppm feed 29 d	TD[c]	Decreased	Tomar et al., 1988
T-2	CD-1, m	$\leq$ 3 ppm feed 3-16 months	TD[c]	No effect	Scheifer et al., 1987
T-2	CBA, f	$\geq$ 0.9 mg/kg/d, po, 21 d	None	Increased	Cooray and Lindahl-Kiessling,1987
DAS	Balb/c, m	0.5-2 mg/kg ip, days 1-3 before, and days 4-7 after antigen	TI[a,b]	Increased	Rosenstein et al., 1981
DON	Balb/c, m	$\geq$ 10 ppm feed 1-2 weeks	TD[c]	Decreased	Robbana-Barnat et al., 1988
3-Acetyl-DON	CD-1, m	10 ppm feed 35 d	TD[c]	Increased	Tomar et al., 1987
FUS-X	Balb/c, m	$\geq$ 25 mg/d daily for 7 d prior to antigen	TD[d]	Decreased	Masuda et al., 1982

TI,thymic independent
TD, thymic dependent
[a]DNP-Ficoll
[b]polyvinylpyrrolidone
[c]SRBC
[d]DNP-ovalbumin

(1984) reported on a superinduction of human IL-2 mRNA following *in vitro* exposure of lymphocytes to T-2 toxin. Il-2, is the acronym for interleukin-2, a growth factor particularly important for the proliferation of T cells. The authors did not quantitate the production or release of IL-2, though they hypothesized that a repressor of mRNA synthesis was affected by T-2 toxin. Though the two cytokines, IL-1 and IL-2, both important in the activation and proliferation of T cells, have been shown to increase *in vitro* (either as product or mRNA), the significance of this observation in the interpretation of the *in vivo*, immunomodulatory activity of various tricothecene mycotoxins has not been forthcoming.

Macrophage Activity. Study of the toxicity of trichothecene mycotoxins on macrophages has been limited to T-2. Many of the known effects of T-2 on mammalian macrophages have been summarized in Table 4. To date, both pulmonary and peritoneal macrophages and polymorphonuclear leukocytes have been evaluated following exposure to T-2. Gerberick and Sorenson (1983) reported that rat alveolar macrophages cultured with 8.93 or 0.33 μM for 20 hr exhibited a fifty percent reduction in cell viability and the number of attached cells, respectively. The authors also reported a significant shrinkage of the cells when incubated in the presence of 0.1 μM T-2. In a separate report, (Gerberick et al., 1984) the authors evaluated various functions of alveolar macrophages subsequent to T-2 exposure. Macromolecular syntheses decreased in the presence of 0.01 μM T-2. Phagocytosis was also affected by *in vitro* T-2 treatment. Both phagocytic capacity and activity were reduced approximately 85%. This effect was apparent after a 20 hr incubation. Additionally, the authors reported that 0.01 μM T-2 inhibited the activation of alveolar macrophages by either LPS or supernatants from PHA stimulated-lymphocyte cultures. Following either *in vivo* or *in vitro* exposure to T-2, Vidal and Mavet (1989) reported a reduction in the phagocytosis of *Pseudomonas aeruginosa* by peritoneal macrophages. However, the clearance of colloidal carbon was reportedly not affected by *in vivo* T-2 treatment. The phagocytosis of *Aspergillus fumigatus* conidia by rabbit alveolar macrophages was reduced in cultures containing serum from T-2 exposed animals (0.5 mg/kg daily for 21 days), however, the ability of alveolar macrophages harvested from T-2-treated rabbits was not affected. Corrier et al. (1987) reported that a single oral exposure to T-2 toxin had no effect on the ability of peritoneal macrophages (*in situ*) to phagocytose sheep red blood cells (SRBCs). The

Table 4. Summary of Trichothecene Mycotoxin Effects on Macrophages.

TOXIN	SPECIES	STRAIN, SEX	DOSE, DURATION	RESPONSE	REFERENCE
T-2	Human		3mg/10^6 cells 20 min	Reduced phagocytosis of opsonized streptococci by PMNs.	Yarom et al., 1984
T-2	Mouse	ICR, f	4 mg/kg, po, single injection	Increased phagocytic activity of peritoneal macrophages in mice treated with T-2 and subsequently sensitized with sheep red blood cells. If toxin exposure was after antigen, sensitization phagocytic activity decreased.	Corrier et al., 1987
T-2	Rabbit	NZW, f	0.5 mg/kg/d, po, daily for 21 d	Phagocytosis of killed *Aspergillus fumigatus* conida by pulmonary macrophages from both vehicle and toxin treated rabbits was reduced only in the presence of serum from T-2 treated animals.	Niyo et al., 1988
T-2	Rat	Long-Evans, m	10^{-7} M, *in vitro*, 6 hr exposure	Phagocytosis of [^{3}H]*Staphylococcus aureus* by pulmonary macrophages was reduced by approximately 20%.	Gerberick et al., 1984
T-2	Rat	Long-Evans, m	$\geq 10^{-8}$ M, *in vitro*, 20 hr exposure	Decreased phagocytosis of *S. cerevisiae* by pulmonary macrophages, a slight reduction of the phagocytosis of *S. cerevisiae* was observed with 10^{-7} M T-2, 6 hr treatment.	Gerberick et al., 1984

effects of T-2 in SRBC sensitized mice, however, varied depending on the temporal relationship of toxin and antigen exposure. When sensitization preceded T-2 treatment, phagocytosis decreased, whereas, post-toxin sensitization resulted in increased phagocytosis. The phagocytosis of opsonised Staphlococcus aureus by human polymorphonuclear cells decreased in cultures exposed to 3 mg T-2 per 10^6 cells for 10 min (Yarom et al., 1984).

SUMMARY

Herein, data suggesting that exposure to trichothecene mycotoxins causes impaired immune function have been reviewed. By way of summary, I would like to provide an alternative explanation for the immunodysfunctions observed subsequent to trichothecene mycotoxin exposure.

Many investigators have pursued the T-cell as the target of trichothecene mycotoxin immunotoxicity. Cortical depletion within the thymus, a reservoir for T cells, is often observed following trichothecene mycotoxin treatment. Ultimately, T-cell imbalance or dysfunction may account for trichothecene mycotoxin-induced immunotoxicity. However, the manifestations of trichothecene mycotoxin-induced immunotoxicity may arise as a culmination of various injuries, as these compounds are systemic toxins, which affect the organisms ability to maintain a normal state of homeostasis. Hence, complex functions, such as mounting an immune response, may be impaired. Presented below are some experimental data in support of this hypothesis.

Exposure to T-2 has been reported to increase the serum levels of cortisol in swine (Rafai and Tuboly, 1982) and corticosterone in mice (Taylor et al., 1989b). Both reports speculated that these hormonal increases may be associated with the immunotoxicity of T-2. The trichothecene mycotoxins are recognized for their ability to induce inflammatory responses in laboratory animals, and the endogenous glucocorticoid hormones (ie. cortisol and corticosterone) play a protective role in mediating these responses. Administering synthetic glucocorticoid hormones to animals subsequently exposed to T-2 decreases T-2 associated lethality (Tremel et al., 1985; Ryu et al., 1987). The glucocorticoid hormones, like their synthetic analog Dexamethasone® are known to be immunosuppressive, and in rodents, thymocytes are particularly sensitive to the lytic effect of these hormones (Claman, 1972). The adrenal glands are the major source of glucocorticoid

hormones, and we have observed (unpublished) that removal of this gland from mice, prior to T-2 treatment, reduced the effect of T-2 on antibody response. The specific adrenal gland product responsible for the disturbance of the immune response in T-2 treated animals remains to be identified, as, in addition to glucocorticoid hormones, there are other adrenal products which influence immune response (e.g. norepinephrine and methionine-enkephalin). Elevated levels of serum norepineprine have been reported in swine following T-2 exposure (Lorenzana et al., 1985).

Recently, it was reported that exposure to T-2 results in the development of endotoxemia in mice (Taylor et al., 1989b). Tai and Pestka (1988b) reported that T-2 treatment decreased the lethal dose of endotoxin in mice. Endotoxin is immunogenic, has adjuvant characteristics in that it can enhance the development of an immune response toward other immunogens, and endotoxin treatment has been shown to decrease the response to sheep red blood cells (reviewed in Jacobs, 1981). In addition, endotoxin is a very potent stimulus of the stress response, ie., activation of the hypothalamic-pituitary-adrenal axis (Carroll et al., 1969). It has stimulatory effects on splenic progenitor cells (Burgess and Nicola, 1983), and causes macrophages to release various cytokines, ie., TNF-α. Thus, the presence of endotoxin in the organism as a consequence of T-2 treatment may be a confounding factor in the assessment of this toxins immunotoxicity.

Based upon the above observations, the following conjecture is made. Though the trichothecene mycotoxins are potent inhibitors of macromolecular synthesis, and though this phenomenon has been demonstrated in immunocytes *in vitro*, the *in vivo* scenario of trichothecene mycotoxin-induced immunotoxicity may not depend entirely on an inhibition of macromolecular synthesis within immunocytes. It is quite probable that alterations of homeostasis, evidenced as increased glucocorticoid hormone levels and the development of endotoxemia may be the causative factors which are ultimately manifested in an alteration of a complex physiologic function, *ie*. immune response.

CONCLUSION

There is no question that the trichothecene mycotoxins have posed, and will continue to pose, a health threat to humans and agricultural animals. The severity of the problem is being dealt with through the development of identification techniques, as well as the potential use of

decontamination strategies. As reviewed by Jelinek et al. (1989), many countries have established exposure limits for several trichothecene mycotoxin contaminants. Herein, data suggesting trichothecene mycotoxins impair an organisms immune system have been reviewed. However, whether or not such compromises in themselves jeopardize an organisms health or productivity is questionable, as, I have indicated, these compounds are potent, systemic poisons.

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The Natural Occurrence, Mutagenic and other Toxicological Implications of Fusarin C from *Fusarium moniliforme*

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INTRODUCTION

The fungus Fusarium moniliforme occurs in both the humid temperate and tropical zones of the world where it is a major parasite of several economically important gramineous plants, especially corn (Zeae mays) (Marasas et al., 1984; Ayers et al., 1989; Leonian, 1932; Kommedahl and Windels, 1981). The fungus consists of a myriad complex of plant pathogenic types, some always virulent, others virulent but only under severe environmental stress, while still others are nonvirulent. It is primarily this latter category which enters the food chain where some isolates are capable of producing one of several known mycotoxins: moniliformin, the fumonisins, and the fusarins. This field fungus, which under favorable circumstances can continue its production of toxins in storage, has been shown to be hepatotoxic (Kriek et al., 1981; Voss et al., 1989) and it is implicated in the etiologies of human esophageal cancer, abnormal bone development of poultry, and equine leukoencephalomalacia (Marasas et al.,1984). Some of its mycotoxins have been identified as producing one or more of these animal disorders (Marasas et al., 1988; Gelderblom et al., 1988). Pure cultures of the fungus are acutely toxic to mice, pigs, sheep, ducklings, and baboons (Kriek et al., 1981; Marasas et al., 1984; Thiel et al., 1986; Jeschke and Nelson 1987).

The involvement of F. moniliforme and its toxins in the clinical picture of specific animal disorders is not completely defined. This is due in part to the very recent interest into the toxicity of this fungus, which is axiomatic because this fungus is one of the earliest known causes of a

mycotoxicosis. As early as 1881 it was established that this fungus, identified as Oospora verticillioides, was the cause of human pellagra, making it second to Claviceps sp. and ergotism, the eariest known example of a mycotoxic species. Within this same time period it was established in this country that this fungus was the cause of horse toxicity. However, only recently, 1988, has the structure of the responsible toxin been established (Gelderblom et al., 1988).

It is our intention to review results of experiments of several laboratories which established that F. moniliforme produces a potent mutagen, fusarin C, and which suggest the possible association of this compound with immunosuppressive activity.

MUTAGENIC ACTIVITY

Bjeldanes and Thomson (1979) were the first to establish that culture extracts from 64% of their isolates of F. moniliforme were mutagenic to the Salmonella typhimurium-microsome system. Of the four tester strains used, the most mutagenic activity was obtained when TA 100 was used in conjunction with hepatic supernatant. In a series of papers it was established that the compound responsible for the mutagenic activity was a 2-pyrrolidone substituted conjugated polyene chromophore which was given the trivial name fusarin C (Figure 1)(Wiebe, and Bjeldanes, 1981; Gelderblom et al., 1984; Gaddamidi et al.,1985). It was then assumed that the cyclic aminol epoxide of the 2-pyrrolidone moiety of fusarin C was responsible for the biological activity. In this regards fusarin A and D are not mutagenic and both lack the epoxide group (Figure 1).

Fusarium crookwellense is the only other Fusarium sp. known to produce this compound (Golinski et al., 1988), and its production by other genera are unknown. A somewhat similar aminol epoxide containing compound, cerulenin, is produced by the fungus Cephalosporium caerulens and it has both antibiotic and antifungal activity (Boekman and Thomas, 1979). The biological activity of cerulenin is due to the fact that it is a potent inhibitor of fatty acid synthesis (Omura, 1976) which possibly acts by inactivating the enzyme complex B-keto-acyl synthetase (Vance et al., 1972). Fusarin C has not been tested for inhibitory activity on fatty acid synthesis, and its activity against fungi and other microorganisms is unknown. In the southeastern USA 95% of all isolates of F. moniliforme produced fusarin C on a liquid medium and on corn (Bacon and Hinton, 1988). The concentration of fusarin C produced by these isolates varied from 7.4 ug/L to 90 ug/L on the test medium

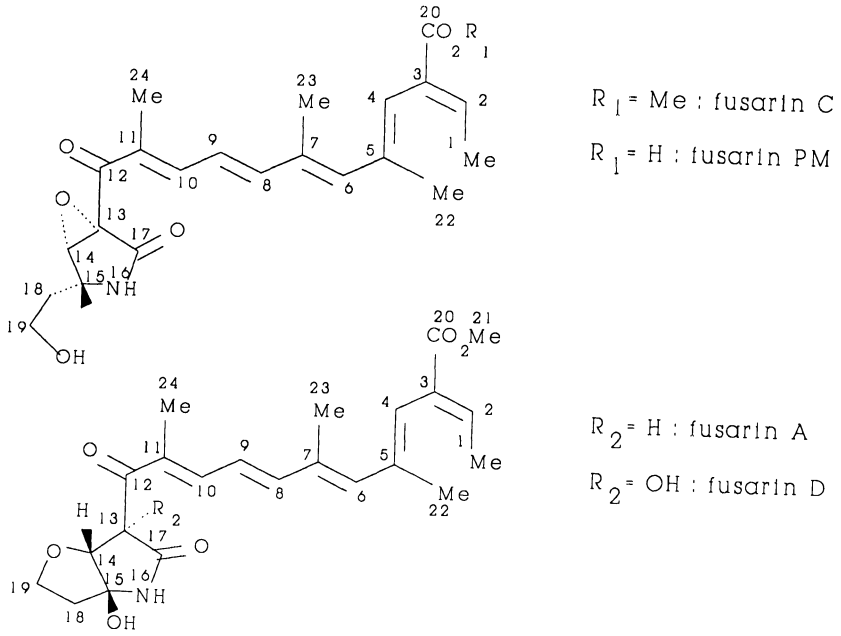

Figure 1. The Chemical Structures of the Fusarins.

of Farber and Sanders (1986). The level of the mutagen produced by an isolate on this medium corresponded with comparable concentrations produced on corn (Bacon et al., 1989). Corn and corn products are not the only food substrate known to be suitable for fusarin C production as it has been experimentally produced on barley, oats, soybeans, and wheat (Table 1) (Bacon et al., 1989). It is not known if fusarin C occurs naturally on these four food stuffs, but it does occur naturally on either F. moniliforme infected, i.e. moldy, or healthy appearing corn in the USA (Thiel et al., 1986), Canada (Farber and Sanders, 1986), China (Cheng et al., 1985), and South Africa (Gelderblom et al., 1984). Thus because of the worldwide occurrence of this compound and production by most isolates, it is important that we establish any animal and human risk to fusarin C.

Table 1. Production of Fusarin C on Autoclaved Cereals and Soybean by an Isolate of F. moniliforme.

Substrate	Fusarin C, ug/g
Corn	147.71[a]
Barley	1028.76
Oats	1267.28
Soybean	98.72
Wheat	636.35

[a]Values are means or three replicates.

A dose-response curve of fusarin C's mutagenic activity in the Salmonella test relative to the mutagenic activity of aflatoxin B_1 and sterigmatocystin indicated that it was not as potent as these two mycotoxins. Fusarin C is mutagenic to the Salmonella strain, TA 100, the base substitution tester strain, and is weakly mutagenic to the frame-shift tester strains. There is no mutagenic activity if a liver microsomal fraction is not included in the assay. Therefore Wiebe and Bjeldanes (1981) concluded that it was a promutagen which is converted to a mutagen in the liver. Gelberblom et al. (1984) established that fusarin C must be activated which depended an all components of the microsomal system. The activated state is water soluble and in the absence of NADPH-generating system, forms a highly soluble derivative, fusarin PM_1 (Figure 1). Both unactivated fusarin C and PM_1 are less mutagenic than activated fusarin C.

Fusarin PM_1 is formed by the action of carboxylesterase which hydrolyzed the C-20 methyl ester of fusarin C to a free carboxylic acid derivative (Gelderblom et al., 1988). Ultraviolet light alters fusarin C, producing three forms which are still mutagenic as only the side chain is modified (Gelderblom et al., 1984). If the five membered ring is altered, the resulting compound may or may not be mutagenic as biological activity depends on whether of not the C_{13}-C_{14} epoxide group is altered. This is demonstrated by the occurrence of spontaneous decomposition products which are non-mutagenic forms of fusarin C that differ only in structural configuration of the 2-pyrrolidone moiety (Gelderblom et al., 1984). Mutagenic activity also is decreased by thiol compounds such as glutathione and cysteine which bind to fusarin C, deactivating it (Gelderblom et al., 1984). These various derivatives of fusarin C have only been reported in pure extracts, their occurrence naturally on solid substrates has not been demonstrated. Fusarin C in the isolated state is not stable, decomposing into unknown products unrelated to those resulting from spontaneous decomposition products (Scott et al., 1986). Furthermore, corn meal spiked with fusarin C and heated to 100 C resulted in partial loss of fusarin C while at 230 C there was a complete loss. However, fusarin C was stable in ground corn when it was stored at room temperature in the dark (Scott et al., 1986).

Fusarin C is also mutagenic to mammalian cells (Cheng et al., 1985). In a cell line there was a significant number of micronuclei induced, an increase in chromosome abberations, and a significant induction in sister chromatid exchanges of cells treated with fusarin C, (10 ug/ml), providing the microsomal fraction was added. At concentrations in excess of 10 mg/ml, fusarin C was toxic to cells without the microsomal fraction and at concentrations of 100 ug/ml it was toxic to cells with or without the microsomal fraction. Fusarin C is also toxic to larvae and adult brine shrimp (Bacon, C.W. unpublished). The mechanism of toxicity in both cases is unknown.

IMMUNOSUPPRESSION

The C_{13}-C_{14} epoxide, located on the pyrrolidone moiety is responsible for the mutagenic properties, but the presence of an epoxide group also implies that fusarin C has carcinogenic properties. Gelderblom et al., (1986) used high and low producing strains of fusarin C to test culture extracts on the mouse skin assay with 12-0-tetradecanoylphorbol-13-acetate.

They concluded that since the high fusarin C strain was not carcinogenic, fusarin C did not act as an initiator of cancer. Their results also indicated that culture extracts of the low produing strain of F. moniliforme were hepatocarcinogenic in rats. Currently the hepatocarcinogenic effects in rats is considered to be due to the fumonisins (Gelderblom et al., 1988).

In examining the role of fusarin C in carcinogenesis Dong and Zhang (1987) presented data which suggested that while fusarin C was not a carcinogen or promoter, it might induce cell mutation and malignant transformations, while also inhibiting that part of the immune system mediated by macrophages. They based their rational on the evidence which indicates the importance of macrophages in host defence to tumor cells and data indicating the influence of several mycotoxins on the immune system (Hibbs, 1972; Friend et al., 1983). Their results showed that activation of mouse peritoneal macrophages treated with purified fusarin C, 6 ug/ml, was strongly inhibited. The inhibition was dose dependent and cytotoxic. Macrophage tumor cytolysis and cytostasis were the most sensitive, 0.6 and 0.5 ug/ml, respectively. Dong and Zhang (1987) also established that the cytotoxic activity of activated macrophage was also inhibited and that the inhibitory effects were reversible but required 72 hr. This indicates that the inhibition by fusarin C is not due to cell death. The mechanisms of inhibition of activated macrophage, and inhibition of macrophage activation are unknown, although Dong and Zhang (1987) suggested that the inhibition of activated macrophages might be due to a disturbance of production and/or release of proteases and H_2O_2, two substances normally secreted by activated macrophages and responsible for cytolysis.

The effects of fusarin C on specific immune responses were determined using an in vitro tumor specific lymphocyte proliferative assay system developed from DBA 12 mice (Chen and Zhang, 1987). In this study it was shown that within 3 to 5 hr fusarin C inhibited lymphoma specific lympho-proliferative responses at 2.5 ug/ml which was lower than the concentration required to induce mutations. The T-lymphocytes and spleen adherent accessory cells are affected. Fusarin C suppression of these cells was restored by exogenous interleukin 2, and at the concentrations used, it was not toxic to the lymphocytes. We re-examined the role of fusarin C on lymphoid cell proliferation by using three cell lines: interleukin 2 dependent CTLL-2 cells, cytochrome-C specific T cell hybridoma, and mouse spleenocytes (Marijanovic et al., 1989). The T cell hybridomas were slightly more sensitive to fusarin C than the interleukin 2 dependent CTLL-2 cells (Table 2 on next page). We concluded that fusarin C is a potent inhibitor of

Table 2. Effects of Fusarin C on Cell Proliferation (Thymidine Incorporation) of Three Cell Lines (Marijanovic et al., (1989)).

Cell Types	Fusarin C, M			
	10^{-1}	10^{-8}	10^{-6}	10^{-3}
	%Thymidine Incorporation			
Cell hybridoma	74	23	10	2
CTLL-2 cells	nd[a]	60	17	1
Mouse spleenocytes	nd[a]	120	47	21

[a]None dectected.

lymphoid cell proliferation or cells undergoing rapid cell division. The precise mechanism of suppression by fusarin C is unknown, but this suggests that it might play an indirect role in carcinogenesis by directly inducing cell mutations, and preventing the destruction of transformed cells by interfering with some aspect of the cell-mediated immunity response.

The possible immunosuppressive effects of F. moniliforme in vivo was examined useing White Leghorn chickens (Marijanovic et al., 1988). These chickens received diets prepared from cultured corn material of four strains of F. moniliforme that differed in the amount of fusarin C produced on corn. After a five week feeding period, immunosuppresant effects were determined by measuring levels of anti-sheep red blood cells and Brucella abortus antibodies. These studies indicated that feed contaminated with F. moniliforme might produce deficiencies in the immune system of chickens, lowering the disease resistance of these animals. Since this study used fungus cultures in the diets, the in vivo effects of fusarin C on this animal, as well as any other species, has yet to be determined.

SUMMARY

Fusarin C is a common secondary metabolite of the majority of isolates of F. moniliforme but whose occurrence and significance in the food and feed chain remain to be established. Since the compound is heat labile, it might not be a human health hazard in cooked foods, although the toxicity of its

decomposition products is unknown. Fusarin C might be more of a problem in animals which is based on its possible role in immunosuppression.

The fungus cultures and isolated fusarin C, in a short term *in vitro* bioassay, showed marked mutagenic activity which is comparable with that of sterigmatocystin and aflatoxin B_1. In addition to being mutagenic it is also immunosuppressive and might play an indirect role in carcinogenesis by interfering with lymphoid cell proliferation. It has been shown that after isolation, fusarin C exists in many forms. If these forms exist *in situ*, any one of them might produce a specific *in vivo* physiological responses in one species, or an entirely different *in vivo* responses in yet other species. Some of these forms are inactive in the bioassays described, thus in the absence of those conditions responsible for the formation of the active forms, the presence of fusarin C in a food stuff might not result in a toxic response.

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PART B

MYCOTOXINS: THEIR DETECTION, OCCURRENCE, INTERACTION, ANALYSIS, AND ECOLOGY

MYCOTOXINS: THEIR DETECTION, OCCURRENCE, INTERACTION, ANALYSIS, AND ECOLOGY

INTRODUCTION

Contamination of agricultural commodities with mycotoxins is overwhelmingly an animal health problem--at least in developed countries. Only in the secondary arena is mycotoxin contamination of agricultural commodities a public health concern. The regulatory agencies with responsibility for minimizing aflatoxins in foodstuffs are necessarily driven by law. This regulation relates to the carcinogenic activity of aflatoxin and, in reality, aflatoxin is only a single facet of a much larger and possibly even more complex scientific field relating to naturally occurring carcinogens and accepatable risks.

The study of mycotoxins--mycotoxicology--is an interdisciplinary field involving animal scientists, plant scientists, mycologists, chemists, toxicologists, animal scientists, agricultural engineers, regulatory agencies, physicians, and veterinarians. Mycotoxicology, which arose as a identifiable field of inquiry in 1960, has focused primarily on aflatoxin which is produced by Aspergillus flavus and A. parasiticus and, more recently, the toxins produced by species in other fungal genera.

The research emphasis in mycotoxicology changes very rapidly as new discoveries are published, but there are several central themes based primarily on the fungi involved in natural outbreaks of mycotoxicoses and the species of domestic animals affected. As would be expected in a relatively new field of inquiry, the mycotoxins which have been described to date only partially explain the signs and symptoms observed in field outbreaks. Currently, the fungal genera given the most attention by mycotoxicologists are: Aspergillus, Penicillium, and Fusarium.

The papers in this section comprise a series of original research

reports, epidemiological studies, or topical summaries of research results compiled by scientists who have a primary interest in mycotoxicology. One of the following papers deals with the ecology of fungal deterioration of stored corn. Also, aflatoxin occurrences are discussed in papers concerned with price support corn and corn meal. Several papers in this section cover state of the art immunochemical and other methods of analysis for aflatoxin and T-2 toxin. Two papers cover some of the aspects of aflatoxin interaction with zinc nutrition in hamsters and cyclopiazonic acid interaction with zinc nutrition in broiler chicks. Three of the papers herein deal with the effects of aflatoxin in swine--two are devoted to the effects of aflatoxin on reproduction in swine, and one documents certain interactive effects observed in swine fed diets contaminated with various combinations of aflatoxin, zearalenone, and T-2 toxin. One paper is a report on the toxicity of seven metabolites of Fusarium moniliforme in four mammalian cell lines. Other papers discuss the occurrence of a metabolite of aflatoxin in milk and the ecology of aflatoxin-producing species in soil.

The papers outlined above cover a broad array of topics reflecting the multi-disciplinary nature of mycotoxicology. The scientists authoring these original reports are well-known and respected by their peers. By participating and sharing these reviewed works, they have enriched the scientific knowledge in mycotoxicology.

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Interactive Effects of Multiple Mycotoxin Contamination of Swine Diets

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INTRODUCTION

Mycotoxin contamination in grains and feedstuffs is a serious agricultural and public health problem. Mycotoxins are causative agents in numerous disease syndromes in swine (Hesseltine, 1979). Over 200 mycotoxins have been identified but relatively few have been confirmed in mycotoxicoses (Tuite, 1979). Aflatoxin B_1 (AFB_1), which is produced by the fungal genus Aspergillus, has been studied the most extensively. Species of the genus Fusarium that produce zearalenone (ZE) and trichothecene toxins (T-2 toxin and deoxynivalenol) are common in many plant materials. At the present time, even a cursory examination of the scientific literature leads to the conclusion that mycotoxins from these two genera are responsible for most of the adverse effects on swine production. Subacute exposure of swine to these toxins adversely affects health, growth, and reproduction (Hintz et al., 1967; Christensen et al., 1972; Chang et al., 1979), all of which are important determinants of profitability.

Most experiments have been designed to study only one mycotoxin, but the simultaneous presence of aflatoxin B_1, zearalenone, and trichothecene toxins in grain has been documented (Hagler et al., 1983; McMillian et al., 1983; Hagler and Behlow, 1984; Hagler et al., 1984; Babadoost et al., 1987). There is a paucity of data to establish whether interactive effects exist between mycotoxins in swine diets. Mycotoxins may have the capacity to interact and thereby increase, decrease, or in other ways modify effects. Therefore, the objective of the present studies was to determine whether interactive effects occur when swine are exposed to dietary T-2 toxin, ZE, or AFB_1.

MATERIALS AND METHODS

Two experiments were conducted using two levels of dietary T-2, ZE, or AFB_1 in a 2 x 2 x 2 factorial arrangement of treatments. Zearalenone and T-2 were produced in pure crystalline form as described previously (Hagler and Mirocha, 1980; Hagler et al., 1981). Crystalline AFB_1 was purchased from Sigma Chemical Company, St. Louis, MO. In each experiment, 80 pigs (2.91 kg average weight) were allotted based on weight, sex, and litter origin at 6 days of age to one of 8 dietary treatments (TRT). The piglets were managed according to standard procedures at birth including ear notching, castration of males, iron shots, and clipping needle teeth and tails. Pigs were housed in an environmentally controlled nursery facility in groups of 10 (5 males and 5 females) in elevated pens with woven wire flooring. The pigs were fed a liquid basal diet (Table 1) at the rate of 2.5 g $DM/hr/kg^{.75}$ using an automated feeding device (Choretime Equipment Co., Milford, IN 46542). This device has been successfully used in previous experiments to examine the nutrient requirements of early weaned pigs (Coffey and Jones, 1985; Jones, 1985). The pig's age is inversely related to susceptibility to mycotoxins (Sisk and Carlton, 1972). The liquid diet eliminated feed refusal problems often encountered with young swine fed mycotoxins and the automatic feeding system provided uniform nutrition varying only in toxin content.

Experiment 1 was conducted to determine the sensitivity of pigs to the levels of added toxins in this system. In Exp. 1 the basal diet was supplemented with 0 or .5 mg/kg T-2, 0 or 1 mg/kg ZE, or 0 or .5 mg/kg AFB_1. In Exp. 2 the basal was supplemented with 0 or 1.5 mg/kg T-2, 0 or 1 mg/kg ZE, or 0 or 1 mg/kg AFB_1.

Pigs were weighed every 4 days during the initial 24 days of the studies (period 1). At the end of period 1 the pigs were transferred to a typical dry corn-soybean meal swine starter diet containing 18% crude protein (CP) and levels of vitamins and minerals to meet NRC (1988) requirements. Pig weights and pen feed consumption were determined weekly over the 14-day dry feeding period (period 2). During period 2 the toxins were added to the feed in Exp. 1, but not in Exp. 2. The toxins were not fed during period 2 in Exp. 2 in order to determine if there were residual effects due to toxin exposure during period 1.

In Exp. 2, at the end of period 1 the pigs were fasted for 6 hr then samples of blood were obtained via jugular puncture. Concentration of plasma cholesterol was determined spectrophotometrically (Gilford Series 252 UV/VIS kinetic recording system, Gilford, Oberlin, OH 44074; Beckman model 2400 monochromator, Beckman Instruments, Inc., Fullerton, CA 92634) by using a cholesterol reagent kit (Stanbio Laboratory, Inc., San Antonio, TX 78202) based on the Lieberman-Burchard reaction, and triglycerides were determined

Table 1. Nutrient Content of the Basal Diet[a].

Nutrient	%
Protein	24.5
Fat	16.0
Carbohydrates[b]	47.3
Vitamin-mineral[c]	2.2
Water	10.0

[a]As is basis.

[b]Corn syrup solids.

[c]Provided vitamin A, 26,400 IU; vitamin D_3, 6,336 IU; vitamin E, 52.8 IU; vitamin K, 13.2 mg; vitamin B_{12}, 79.2 μg; riboflavin, 15.6 mg; d-pantothenic acid, 52.8 mg; niacin, 105.6 mg; choline chloride, 1.85 g; folic acid, 1.59 mg; d-biotin, .26 mg; zinc, 60 mg; iron, 660 mg; manganese, 332.4 mg; copper, 52.8 mg; phosphorous, 1.7 g; iodine, 6.6 mg; cobalt, 2.64 mg; selenium, 1.58 mg.

by using a kit (Sigma Diagnostics Triglycerides, 340 nm, Sigma Chemical Co., St. Louis, MO 63178) based on the ultraviolet procedure of Bucolo and David (1973). Plasma urea was analyzed using the modified assay of Chaney and Marbach (1962) and protein by the procedure of Lowrey et al. (1951). Vitamins A and E were determined according to the procedures of Catignani and Bieri (1983), and aflatoxin B_1 residues were determined by the method of Hutchins et al. (1988). Two male pigs (closest to the average weight) from each treatment group were killed by exsanguination, and their livers were removed. The livers were sampled for analysis of dry matter and ether extract, according to the standard AOAC procedures (1984), and aflatoxicol content (Tyczkowska et al., 1987).

Data were analyzed as a randomized design containing a 2 x 2 x 2 factorial arrangement of treatment (Steel and Torrie, 1980) using least-squares analysis of variance according to the General Linear Models procedure of the Statistical Analysis System (SAS, 1982). If the F-test was significant ($p<.05$), single degree of freedom contrasts were used to determine differences between levels and significant interaction were examined by fitting the main effects separately in the model.

RESULTS

In Exp. 1 there was a T-2 x ZE x AFB_1 x sex interaction ($p<.05$) on average daily gain (ADG) during periods 1 and 2 and over the entire study (Table 2). Feeding T-2 or ZE alone or in combination (TRT 3, 5, and 7) did

not alter growth. During period 1, growth of females, but not castrated males was depressed ($p<.05$) by feeding .5 mg/kg AFB_1 (TRT 2), but feeding 1 mg/kg ZE in combination with AFB_1 (TRT 4 and 8) alleviated the growth depressing effect of AFB_1 on the female pigs. During period 2 feeding AFB_1 alone (TRT 2) depressed ($p<.05$) the growth rate of both sexes, but the depression was greater ($p<.05$) in females. As in period 1, the interaction of 1 mg/kg ZE in combination with .5 mg/kg AFB_1 (TRT 4 and 8) overcame the growth depression caused by AFB_1 alone. Over the entire study, feeding AFB_1 alone or in combination with T-2 depressed ($p<.05$) the growth of females (TRT 2 and 6), but feeding AFB_1 in combination with ZE (TRT 4 and 8) resulted in gains that were similar to those of females fed the control (TRT 1).

In Exp. 2 there were no interactions by sex; therefore, the data are presented as the mean of both sexes (Table 3). There was a T-2 x ZE x AFB_1 interaction ($p<.05$) on ADG during period 1. Feeding 1 mg/kg AFB_1 alone (TRT 2) or in combination with T-2 (TRT 6) reduced ($p<.05$) ADG compared to the diet containing no added toxins (TRT 1). However, feeding 1 mg/kg ZE in combination with AFB_1 (TRT 4 and 8) resulted in a moderate increase in growth rate that was greater ($p<.05$) than pigs fed only AFB_1, but less ($p<.05$) than pigs fed the basal with no added toxins. Feeding ZE or T-2 alone or in combination (TRT 3, 5, and 7) caused only a slight reduction in growth.

There was an AFB_1 effect on ADG ($p<.05$) during period 2. Toxins were not added to the dry diet in Exp. 2 in order to determine the extent of recovery to toxin exposure. Pigs that were fed AFB_1 during period 1 continued to be affected by toxin exposure and grew more slowly ($p<.05$) during period 2.

Plasma metabolites were altered in a manner characteristic of aflatoxin exposure (Table 3). There were reductions ($p<.05$) in plasma concentration of vitamins A and E as a result of feeding AFB_1, regardless of whether other toxins were included in the diet. Plasma triglycerides, protein, and urea were not sensitive to the dietary treatments whereas cholesterol tended to be lower in pigs fed AFB_1. Aflatoxin could only be detected in plasma of pigs fed AFB_1.

Ether extract content was increased ($p<.05$) and dry matter was decreased ($p<.10$) in liver tissue from pigs fed AFB_1 (Table 3). The metabolite of AFB_1, aflatoxicol, was present only in livers of pigs fed dietary AFB_1, and its level was not significantly ($p>.05$) altered by the presence or absence of other toxins in the diet.

DISCUSSION

Interactive effects of mycotoxins on swine have not been previously

Table 2. Effect of Treatment on Swine Growth, Exp. 1[a].

	Treatment								
Toxin, ppm	1	2	3	4	5	6	7	8	
T-2	0	0	0	0	.5	.5	.5	.5	
ZE	0	0	1	1	0	0	1	1	
AFB_1	0	.5	0	.5	0	.5	0	.5	SE[b]
Period 1[c]									
Gain, kg/day[d]									
Male	.17	.20	.19	.20	.21	.18	.20	.18	.005
Female	.21	.17	.19	.23	.20	.19	.21	.20	.005
Mean	.19	.18	.19	.21	.21	.19	.20	.19	.010
Period 2[c]									
Gain, kg/day[d]									
Male	.31	.24	.34	.30	.30	.22	.29	.27	.012
Female	.38	.18	.33	.34	.36	.22	.36	.30	.012
Mean	.34	.21	.33	.32	.33	.22	.32	.29	.024
Overall[c]									
Gain, kg/day[d]									
Male	.22	.21	.24	.18	.24	.19	.23	.21	.007
Female	.28	.17	.24	.27	.26	.21	.26	.24	.007
Mean	.25	.19	.24	.22	.25	.20	.25	.23	.014

[a]Least squares means.

[b]SE = standard error.

[c]Period 1 = liquid feed for 24 days; period 2 = dry feed for 14 days; overall = combined liquid and dry feed periods.

[d]T-2 x ZE x AFB_1 x sex interaction ($p<.05$).

reported; however, Côté et al. (1985) could not rule out the possibility of interaction between dietary deoxynivalenol and zearalenone. The present data confirm that there are interactive effects between mycotoxins and swine performance. The interaction between ZE and AFB_1 was a consistent response. In both Exp. 1 and Exp. 2 feeding .5 mg/kg ZE stimulated growth rate of pigs fed AFB_1. The mechanism for this interaction is not known. The majority of research concerning the effects of ZE on swine have focused on alterations in reproductive performance rather than growth rate. Smith (1980) reported

Table 3. Effect of Treatment on Swine Growth and Plasma Metabolites and Liver Composition, Exp. 2[a].

	Treatment								
Toxin, ppm	1	2	3	4	5	6	7	8	
T-2	0	0	0	0	1.5	1.5	1.5	1.5	
ZE	0	0	1	1	0	0	1	1	
AFB_1	0	1	0	1	0	1	0	1	SE[b]
Period 1[c]									
Gain, kg/day[d]	.24	.15	.20	.17	.19	.16	.20	.17	.02
Period 2[c]									
Gain, kg/day[d]	.30	.21	.33	.22	.33	.26	.36	.25	.21
Plasma									
Vitamin A μg/dl[e]	24.1	6.3	22.1	4.3	17.5	5.9	18.0	3.1	3.2
Vitamin E μg/dl[e]	159.0	130.7	181.4	107.8	181.1	140.7	161.4	157.2	23.9
Cholesterol mg/dl	176.3	164.0	172.0	165.5	137.2	188.4	174.9	161.9	9.5
Triglycerides mg/dl	63.3	75.4	66.7	73.5	76.4	78.4	69.1	63.2	7.7
Protein, g/dl	58.2	59.6	60.2	64.1	64.0	56.4	58.2	55.5	2.2
Urea, mg/dl[d]	2.4	2.2	2.5	2.5	2.4	3.4	2.4	2.5	.15
AFB_1, ng/ml[e]	.0	.4	.0	.4	.0	.7	.0	1.7	.17
Liver									
Dry matter,%[f]	25.6	24.7	26.0	24.4	27.1	25.7	26.8	23.9	1.2
Ether extract %[f]	5.89	7.34	5.55	7.15	4.54	7.57	4.91	7.10	.59
Aflatoxicol ng/g[f]	.00	.35	.00	.11	.00	1.27	.00	.37	.40

[a]Least squares means.

[b]SE = standard error.

[c]Period 1 = liquid feed for 24 days; period 2 = dry feed for 14 days.

[d]T-2 x ZE x AFB_1 ($p<.05$).

[e]AFB_1 effect ($p<.05$).

[f]AFB_1 effect ($p<.10$).

that feeding 50 mg/kg ZE to 5-wk-old pigs resulted in non significant ($p>.05$) depressions in growth rate and feed efficiency but a significant ($p<.05$) uterine enlargement.

Anabolic effects of zearalenone have been reported. Injection of rats with low doses of zearalenone (20-40 μg) was shown to increase body weights, but growth was reduced at higher doses (40-650 μg) (Mirocha et al., 1967). Mirocha et al. (1968) examined the myotrophic activity of zearalenone in mice and reported stimulation of perineal muscle growth. The mechanism for this action is perhaps due to the binding of ZE to estradiol-17-ß binding sites (Boyd and Wittliff, 1978) and promoting translocation of the nuclear receptor (Kiang et al., 1978). The metabolite zearalenol exhibits 3 to 4 times greater activity than zearalenone (Mirocha et al., 1979). Zearalenone was shown to be converted to zearalenol in the rat liver (Olsen et al., 1981).

The growth depression that resulted from AFB_1 was expected; however, the level of AFB_1 required to consistently depress gains is not known. Factors including levels of dietary nutrients are known to interact with severity of AFB_1 toxicity in swine (Coffey et al., 1985). To our knowledge, there are no previous reports of controlled studies to examine the effects of AFB_1 in pigs as young as those used in these experiments. In Exp. 1 the growth depressing effect of AFB_1 was less severe in pigs being fed the liquid diet (period 1) compared to those on the dry diet (period 2). The difference may be due to the nutrient content of these diets. Previous studies have shown that in poultry (Smith et al., 1971) and swine (Sisk and Carlton, 1972; Coffey et al., 1985; Coffey et al., 1987) increasing dietary protein or essential amino acids diminished the growth depressing effects of AFB_1. The liquid diet contained 24% CP (1.47% lysine) compared with 18% CP (.95% lysine) in the dry diet.

In Exp. 1, there was a differential response to .5 ppm AFB_1 due to sex, which resulted in greater growth depression of females than castrated males. This has not been previously observed, but Côté et al. (1985) reported more severe depression in the weight gain of castrated males than females fed deoxynivalenol. The reasons for these differential responses are not known. In Exp. 2, AFB_1 also reduced ADG, but feeding the higher level (1 vs .5 mg/kg) resulted in more severe growth rate reduction that was uniform regardless of sex.

Alteration in concentrations of lipid components measured in the plasma during Exp. 2 are indicative of aflatoxicosis. Lipid malabsorption occurred in poultry during aflatoxicosis due to decreased pancreatic lipase production (Hamilton, 1977). Blood levels of triglycerides, phospholipid-esterified cholesterol, and fat soluble vitamins were lowered by AFB_1 (Hamilton, 1977; Hamilton et al., 1972). It has been proposed that the effect of AFB_1 on lipid

metabolism is the primary lesion during aflatoxicosis (Tung et al., 1972).

The liver is a primary site of AFB_1 damage. Alteration of the integrity of hepatic cellular membranes have been reported previously. Increased liver lipid content from pigs exposed to AFB_1 in the present study is consistent with previous reports (Edds, 1979) and was likely related to the growth depression that continued during period 2 (Exp. 2). Sisk and Carlton (1972) reported hepatocellular necrosis, hemorrhage, biliary hyperplasia, and fatty degeneration of hepatocytes as a result of feeding aflatoxin to 10-wk old pigs.

These data reveal another important determinant in the response of swine to mycotoxins. The level of AFB_1 required to consistently produce a depression in swine performance has not been clearly established. Allcroft and Carnaghan (1963) reported that .2 mg/kg AFB_1 would reduce growth and efficiency of growing swine; in contrast others have concluded that .3 to .4 mg/kg were required to consistently depress performance (Carnaghan and Crawford, 1964; Hintz et al., 1967; Duthie et al., 1968; Monegue, 1977; Southern and Clawson, 1979). The demonstration of interactive effects may help explain some of the divergence of reports concerning the effects of mycotoxin contamination on swine performance.

SUMMARY

Two experiments were conducted to determine whether interactive effects occur when swine are exposed to dietary T-2 toxin (T-2), zearalenone (ZE), or aflatoxin B_1 (AFB_1). In each experiment 80 pigs (2.91 kg average weight) were allotted at 6 days of age to one of 8 dietary treatments (TRT). During the initial 24 days of the studies (period 1), the pigs were fed a liquid basal diet at the rate of 2.5 g DM/hr/$kg^{.75}$ using an automated feeding device. At the end of period 1, the pigs were fed a corn-soy diet (18% CP) for an additional 14 days (period 2). In Exp. 1 the basal diet was supplemented with 0 or .5 mg/kg T-2, 0 or 1 mg/kg ZE, or 0 or .5 mg/kg AFB_1. In Exp. 2 the basal diet was supplemented with 0 or 1.5 mg/kg T-2, 0 or 1 mg/kg ZE, or 0 or 1 mg/kg AFB_1. The toxins were added to the feed during period 2 in Exp. 1, but not in Exp. 2. In Exp. 1 there was a T-2 x ZE x AFB_1 x sex interaction ($p<.05$) on average daily gain for periods 1 and 2 and over the entire study. During period 1 growth of females, but not castrated males, was depressed ($p<.05$) by feeding .5 mg/kg AFB_1; but feeding 1 mg/kg ZE in combination with AFB_1 prevented growth depression by AFB_1. In period 2, AFB_1 depressed ($p<.05$) the growth of both sexes, but the effect was greater ($p<.05$) in females. Feeding ZE during period 2 prevented reduced growth of pigs fed AFB_1. Over the entire study, feeding AFB_1 alone or combined with T-2 reduced ($p<.05$) growth of females, and feeding ZE in combination with AFB_1 alleviated the

growth depressing effect of AFB_1. In Exp. 2 there was a T-2 x ZE x AFB_1 interaction ($p<.05$) on ADG. Feeding AFB_1 alone or combined with T-2 reduced ($p<.05$) growth rate. However, feeding pigs diets containing ZE and AFB_1 resulted in an intermediate growth rate that was faster ($p<.05$) than pigs fed only AFB_1, but slower ($p<.05$) than pigs fed the basal with no added toxins. Pigs fed AFB_1 during period 1 continued to grow slower ($p<.05$) after toxins were removed from their diets during period 2. In Exp. 2, a plasma sample was obtained at the end of period 1, and two pigs from each treatment were killed to obtain liver samples. There were reductions ($p<.05$) in plasma concentration of vitamins A and E in pigs fed AFB_1 regardless of whether other toxins were fed. Plasma triglycerides were not affected by the treatments, but cholesterol tended to be lower in pigs fed AFB_1. Liver ether extract was higher ($p<.05$) in pigs fed AFB_1. These results demonstrate interactive effects between mycotoxins on swine performance.

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The Effects of Aflatoxin-Contaminated Corn on Brood Sows and their Litters from Farrow to Finish

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INTRODUCTION

Aflatoxins are a closely related group of secondary metabolites produced by the fungi Aspergillus flavus and Aspergillus parasiticus (Diener and Davis, 1969). The invasion of feed grains such as corn before, during, and after harvest by these fungi, coupled with poor drying conditions, insect damage and improper storage, promotes aflatoxin production. Aflatoxin contamination of corn has had a serious economic impact upon both the corn and swine producer in the southern and mid-west United States (Nichols, 1983). The greatest economic loss for the swine producer has been due to the adverse affects that the aflatoxins have on growth and feed efficiency, as well as, poor body condition and death (Smith et al., 1976).

The effects of aflatoxin on brood sows have been reported by several workers. Armbrecht et al. (1972) fed diets containing various levels of aflatoxin metabolites to sows throughout four successive parities and found no effect on sow productivity, but there was a weight reduction in sucking pigs. Hintz et al. (1967) fed diets containing 450 μg/kg of aflatoxin B_1 and reported no reproductive abnormalities, nor were there any effects on the growth of the nursing pig.

There have been conflicting reports concerning the effect of aflatoxin on growth and feed efficiency of nursery pigs and growing-finishing swine. Monegue et al. (1977) was unable to show any adverse affects of aflatoxin on the growth and feed efficiency of 9.5 kg nursery pigs with aflatoxin levels up to 300 μg/kg. Other investigators (Duthrie et al., 1966; Southern and

Clawson, 1979) have shown that levels of aflatoxins ranging between 280 and 600 μg/kg resulted in substantial reduction in growth rate and sometimes feed efficiency.

There appears to be a great deal of variation in results obtained from feeding culture-derived concentrates as compared to naturally-contaminated feed sources of aflatoxin to swine. Since the economic losses by the swine producers are due to naturally-contaminated feed sources, the present research was designed to investigate the effects of naturally-contaminated corn containing aflatoxin on sow productivity, piglet performance, and the long term effects on growing-finishing swine.

MATERIALS AND METHODS

Preparation of Aflatoxin Contaminated Corn Diets

Naturally-contaminated corn containing 2.27 ppm of aflatoxin was blended with corn containing less than 20 μg/kg of aflatoxin to yield aflatoxin contaminated diets. Of the aflatoxin metabolites contained in the naturally-contaminated corn, 80% was aflatoxin B_1 and 20% was B_2. Aflatoxin G_1, G_2, T-2 toxin, deoxynivalenol, zearalenone, or ochratoxin A were not detected. Corn and mixed feed samples were taken at the time of mixing using the guidelines of Davis et al. (1980). Aflatoxins were determined by the method of Horwitz (1980).

Sow Study

Diets containing 500 and 750 μg/kg of aflatoxin and a control diet (Table 1) containing less than 20 μg/kg of aflatoxin were fed to three groups of nine sows each throughout two successive parities. Measures of sow productivity such as number of pigs born alive, number of stillborn pigs, birth weight, and litter size at 21 days were recorded. Milk samples (100 ml) were taken at birth and at weaning and analyzed for aflatoxin M_1 (Tyczkowska et al., 1984). The sows were slaughtered approximately 55 days after rebreeding and the reproductive tracts examined for the number of embryos and general tract condition.

Liver and kidney samples, taken from each sow at the time of slaughter and from stillborn pigs and pigs that died during lactation, were assayed for the presence of aflatoxins B_1, M_1, and aflatoxicol (Tyczkowska et al., 1987). Piglet liver and kidney samples were individually extracted and extracts combined to yield samples representing approximately 50 g of wet tissue.

Nursery Studies

An automatic feeding device (Lecce and Coalson, 1976) was used to examine baby pig performance independent from the sow. Milk diets (Table 4) containing 0, 10, 100, and 200 μg/kg of aflatoxin B_1 (Fischer) were fed to four groups of nine pigs each for 24 days. These pigs had nursed the sows

Table 1. Composition of Experimental Diets[a].

Ingredient	Sow Diets[b]	Grower-Finisher Diets[c]		
Crude Protein, %	14	18	16	14
Corn	83.0	72.0	77.1	82.1
Soybean Meal, 44%	14.0	25.2	20.1	15.1
Dynafos	1.5	--	--	--
Calcium Diphosphate	--	1.1	1.1	1.1
Limestone	0.6	0.6	0.6	0.7
Salt	0.4	0.3	0.3	0.3
Vitamin and Mineral Mix[d]		0.4	0.3	0.3 0.3
Antibiotic[e]	--	0.5	0.5	0.5

[a]Naturally-contaminated aflatoxin corn (2.27 ppm) was blended with corn containing less than 20 μg/kg aflatoxin to produce diets containing various levels of aflatoxin.

[b]Sows were fed 1.82 to 2.27 kg feed/day during gestation and 6.35 to 7.26 kg feed/day throughout lactation.

[c]18% diets were fed from 5 to 14 kg; 16% diets from 14 to 56 kg; and 14% diets from 56 to 100 kg.

[d]The vitamin and mineral premixes contained: Vitamin A, 2,000,000; Vitamin D, 480,000; and Vitamin E, 4,000 USP units per pound. Riboflavin, 1,200; d-Panthothenic Acid, 4,000; Niacin, 8,000; Vitamin B_{12}, 6; Vitamin K, 1,000; Choline Chloride, 140,000; and Folic Acid, 120 mg/pound. Trace minerals in percent are Zinc, 5.0; Iron, 5.0; Manganese, 2.50; Copper, 0.4; Iodine, 0.05; Cobalt, 0.02; and Selenium, 0.004.

[e]Tylan Sulfa, Elanco.

for 12 hours to ensure adequate intake of colostrum before being weaned and placed on the automatic feeding device. Rates of gain and average daily gain were measured every two days in order to determine the correct volume of diet necessary for the liquid feeder. Baby pigs from each of the three sow treatments were distributed equally across the 4 milk diets in order to examine any affects on growth which could be related to the sows' dietary treatment during gestation. Blood samples (10 ml) were taken at 21 days of age and analyzed for sodium, potassium, carbon dioxide, chlorine, glucose, blood urea nitrogen, albumin, alkaline phosphatase, and serum glutamate-oxaloacetate transaminase using a Technicon blood analyzer.

Twenty-two of these pigs were used for liquid and dry milk diet studies for an additional 4 weeks. The milk diet, which could be fed in either a dry or liquid form to weanling pigs was prepared (Table 4) according to the methods described by Efird et al. (1982). Aflatoxin B_1 was added to the control diet to provide a diet containing 1000 μg/kg of aflatoxin B_1 (Fischer). Dry diets were provided ad libitum to some (12) of these pigs while others (10) were given liquid diets fed hourly. The liquid diets were fed to provide 60 g of dry matter/kg pig/day. The liquid diet was dispensed at the rate of 12.5 ml/hour/kg pig (20% dry matter) by an automatic feeding device. Rates of gain and average daily gain were measured every four days in order to determine the correct volume of diet necessary for the liquid feeder. Blood samples (10 ml) were taken from the carotid artery or the jugular vein of each pig in this study and assayed for retinol and alpha-tocopherol by the procedure of Bieri et al. (1979).

One hundred of the pigs which had nursed the sows for 21 days were weaned and fed corn-soybean meal based diets for 28 days in the nursery. These diets contained <20 (control), 100, 300, and 500 μg/kg of aflatoxin from the naturally-contaminated corn. Five pigs were allotted by weight per pen with at least one pig from each sow treatment in each pen. Blood samples (10 ml) were taken at 49 days of age (end of trial) and analyzed for retinol and alpha-tocopherol (Bieri et al., 1979).

Finishing Studies

Thirty-two boars and gilts from the nursery study (corn-soybean meal diets) were reared to a final weight of 100 kg. Throughout the nursery and finishing periods, those pigs weaned from sows fed aflatoxin (500 or 750 μg/kg) were fed corn-soybean meal diets containing 500 μg/kg of aflatoxin. Pigs weaned from sows fed the control diet were maintained on control (<20 μg/kg) feed during the nursery and finishing periods. Plasma samples (10 ml) were taken at 159 days of age and analyzed for retinol and alpha-tocopherol (Bieri et al., 1979).

RESULTS

Sow Studies

In neither sow study 1 nor 2 were significant differences observed in the number of pigs born alive, number of stillborn pigs, birth weight, or litter size at 21 days of age (Table 2). However, the mortality rate in the first farrowing study was markedly increased in those piglets sucking sows fed 750 μg/kg of aflatoxin when compared to piglets from control sows. Mortality increased from 7.98% for controls to 17.66% for pigs from sows fed 750 μg/kg of aflatoxin. Mortality was also increased in the second study, but not as dramatically as in the first.

Table 2. The Effects of Feeding Aflatoxin Throughout Gestation and Lactation on Sow and Pig Performance.

Criteria	Sow Treatment (μg/kg Aflatoxin)		
	Control (<20)	500	750
		Sow Study 1[1]	
Pigs born alive/sow	10.20 (1.10)[2]	11.20 (1.50)	11.20 (1.10)
Birth weight, kg	1.33 (0.07)	1.20 (0.06)	1.30 (0.06)
21-day weight, kg	5.37 (0.65)	4.80 (0.55)	4.67 (0.62)
Daily Gain, kg	0.19 (0.01)	0.17 (0.01)	0.16 (0.01)
Mortality, %	7.98 (5.97)	13.66 (6.71)	17.66 (9.82)
Milk aflatoxin content			
Birth, μg M_1/L	0.00 (0.00)	0.05 (0.02)	1.72 (1.15)
Weaning, μg M_1/L	0.00 (0.00)	0.11 (0.05)	0.10 (0.09)
		Sow Study 2[3]	
Pigs born alive/sow	9.40 (0.70)	8.89 (1.50)	11.30 (0.60)
Birth weight, kg	1.41 (0.03)	1.25 (0.03)	1.35 (0.03)
21-day weight, kg	5.34 (0.17)[a]	4.88 (0.16)	4.06 (0.14)[a]
Daily gain, kg	0.17 (0.01)[a]	0.17 (0.01)	0.13 (0.01)[a]
Mortality, %	5.46 (1.04)	3.75 (0.79)	8.73 (4.20)
Milk aflatoxin content			
Birth, μg M_1/L	0.08 (0.05)	0.37 (0.07)	0.66 (0.14)
Weaning, μg M_1/L	0.02 (0.01)	0.04 (0.02)	0.07 (0.02)

[1]5 sows per treatment.

[2]Standard error.

[3]5 sows on control, 7 sows on 500, and 6 sows on 750.

[a]Means with the same superscript are significantly different, $p<.01$.

A summary of the reproductive tract condition at slaughter showed that of the nine sows fed the control diet, five were pregnant while four had returned to estrus. Of nine sows fed 500 μg/kg of aflatoxin, one was pregnant and six returned to estrus. The other two sows exhibited signs of anestrus. The results from the nine sows fed 750 μg/kg of aflatoxin showed that four were pregnant, four returned to estrus, and one had not returned to estrus. There were no differences in the number of embryos, follicles, or corpus lutea across sow treatments.

Overall, 37% of the animals bred were pregnant, 52% returned to estrus, and 11% were anestrus. Of the two sows fed 500 μg/kg aflatoxin that were anestrus, one was in extremely poor body condition and the other had considerable edema in the uterus. The sow which did not recycle and had been fed 750 μg/kg aflatoxin, had abscesses in the pelvic region. Since heat stress is known to affect conception in swine, this could explain the high percentage of sows returning to estrus since these sows were rebred in the month of July.

In the first sow study, aflatoxin M_1 was not found in the milk of control sows (Table 2). In sows fed 500 μg/kg of aflatoxin, the levels of aflatoxin M_1 contained in the milk at birth and weaning were similar. In sows fed 750 μg/kg of aflatoxin, values for aflatoxin M_1 were higher than the 500 μg/kg sow milk, and the level of M_1 was lower at 21 days than at farrowing.

In the second sow study, a low level of aflatoxin M_1 was found in the milk of control animals. This was due to an unexplained increase of aflatoxin in the control diet, from 20 μg/kg to 50 μg/kg, just prior to farrowing. However, as the level of aflatoxin was increased in the sows diet to 500 and 750 μg/kg, the level of aflatoxin M_1 increased in sow milk at birth and weaning. As seen in the first farrowing study, the level of aflatoxin M_1 was lower at weaning than at the time of farrowing.

In the first sow study, as the level of aflatoxin increased in the sow's diet, average daily gain and average 21 day weights of the pigs diminished (Table 2). Pigs that nursed sows fed 500 μg/kg of aflatoxin weighed 11% less than controls at 21 days and the average daily gain was also reduced by 11%. Pigs nursing sows fed 750 μg/kg aflatoxin weighed 13% less at 21 days of age and gained 17% less than control pigs.

In the second study, those pigs sucking sows that were fed 500 μg/kg of aflatoxin had an average daily gain of 173 g which was similar to control pigs. The pigs that sucked sows fed 750 μg/kg of aflatoxin had significantly ($p<.05$) lower average 21 day weights and average daily gains when compared to pigs from control sows.

Aflatoxin M_1 was found in liver and kidney tissues of sows fed the control diet as well as the diets containing 500-750 μg/kg of aflatoxin (Table 3). The highest amount of M_1 was found in liver tissue of a sow fed a diet containing 500 μg/kg of aflatoxin. The amount of aflatoxin found in kidney tissue was much lower (0.0-0.3 μg of M_1/kg tissue) than that found in liver and did not vary among treatment groups.

Trace quantities of aflatoxicol were detected in only one liver sample and one kidney sample from different control sows, but could not be detected in kidney or liver tissues from any of the sows fed diets containing 500 and 750 μg/kg of aflatoxin. Also, a trace quantity of aflatoxin B_1 was found in

Table 3. The Presence of Aflatoxicol and Aflatoxin M_1 in Swine Tissues Based on the Dietary Level of Aflatoxin Fed to the Sow[1].

	Sow Treatment					
	Control (<20)		500		750	
Tissue	X	Range	X	Range	X	Range
			Sow Tissue			
Liver, Af M_1[2]	0.30	0.00-1.00	0.70	0.10-1.70	0.50	0.20-0.80
Kidney, Af M_1	0.04	0.00-0.20	0.20	0.00-0.30	0.20	0.10-0.30
			Stillborn Pig Tissue[4]			
Liver, Af M_1	0.10	0.00-0.25	1.30	--[5]	0.32	0.01-0.79
Liver, Afl[3]	0.60	0.30-0.90	1.15	0.60-1.70	0.56	0.30-0.90
Kidney, Afl	0.85	0.40-1.30	2.10	1.20-3.60	0.55	0.30-0.80
			Pigs That Died During Lactation[4]			
Liver, Af M_1	0.16	0.00-0.92	0.19	0.01-0.64	0.35	0.00-1.36
Kidney, Af M_1	0.07	0.00-0.23	0.05	0.01-0.09	0.14	0.10-0.16
Liver, Afl	0.68	0.30-1.20	0.71	0.30-1.20	0.64	0.30-1.50
Kidney, Afl	0.64	0.30-1.10	0.50	0.20-0.80	0.66	0.30-1.10

[1]All concentrations are listed as μg toxin/kg of wet tissue.

[2]Af M_1 = aflatoxin M_1.

[3]Afl = aflatoxicol.

[4]Tissue extracts were pooled to yield samples representing 50 g of wet tissue. Aflatoxin M_1 was found in two kidney samples from stillborn pigs. One was from a 500 μg/kg sow and the other from a 750 μg/kg sow with the average level of M_1 being 0.07 and 0.08, respectively.

[5]Only one sample representing 2 pigs.

the kidney of one control sow and one sow fed the diet containing 500 μg/kg of aflatoxin. Neither aflatoxicol or aflatoxin B_1 were detected in tissue samples obtained from sows fed diets containing 750 μg/kg aflatoxin.

Aflatoxin M_1 was found in the liver of all stillborn pigs regardless of the dietary regime of the sow (Table 3) and was present in kidney tissue of those stillborn pigs derived from sows that received 500 and 750 μg/kg of aflatoxin in the diet during gestation. Aflatoxicol, on the other hand, was found in both liver and kidney tissue in all stillborn pigs.

Nursery Studies

Piglets fed 10 μg/kg of aflatoxin B_1 weighed and gained similarly to control piglets (data not shown). However, pigs fed 100 and 200 μg/kg of aflatoxin B_1 showed reduced 24-day weights as compared to control pigs (5.04 vs. 4.95 and 4.54 kg for controls, 100 and 200 μg/kg dietary aflatoxin, respectively). The average daily gain was likewise reduced ($p<.05$) when comparing pigs fed 200 μg/kg aflatoxin to those pigs fed a control diet (130 vs. 160 g/day, respectively).

The effects of feeding aflatoxin during gestation on subsequent performance of piglets are shown in Table 4. Pigs from control sows and sows fed 500 μg/kg of aflatoxin performed similarly. However, pigs from sows fed 750 μg/kg of aflatoxin had significantly lower 24-day weights and average daily gains when compared to either pigs from sows fed a control diet or a diet containing 500 μg/kg of aflatoxin.

Table 4. Growth of Pigs Reared in an Automatic Feeding Device[1] Based on the Level of Aflatoxin Fed to the Sow.

	Sow Treatment (μg/kg Aflatoxin)		
Criteria	Control (<20)	500	750
No. of pigs	12	12	12
Birth weight, kg	1.44 (0.07)	1.31 (0.07)	1.25 (0.05)
24-day weight, kg	5.17 (0.22)[a]	5.21 (0.22)[b]	4.53 (0.18)[a,b]
Daily gain, g	160 (10)	160 (10)[a]	140 (10)[a]

[1] Diet composed of Dietrich's dry whole milk, 10% vitamin and mineral premixes, and 79% water. The vitamin premix provided 11,000 IU vitamin A, 1,100 IU vitamin D, 55 mg vitamin E, 10 mg vitamin K, 2.6 mg thiamine, 6 mg riboflavin, 44 mg niacin, 26 mg pantothenic acid, 44 μg vitamin B_{12}, 3 mg vitamin B_6, 0.2 mg biotin, 1.2 mg folic acid, and 1,100 mg choline. the mineral premix provided 0.0027% copper, 0.068% iron, 0.0088% iodine (as iodized salt, 0.01% KI), 0.0091% manganese, 0.0001% selenium, and 0.045% zinc.

[a,b] Means with the same superscript are significantly different, ($p<.05$).

In the study where the automatic feeding device was used, plasma analysis (data not shown) revealed that animals fed aflatoxin B_1 had lower potassium levels as compared to controls (6.9 mmoles K/L) with the reduction being significant ($p<.05$) for pigs fed 10 (6.2 mmoles K/L) and 200 μg/kg (5.9

mmoles K/L). Pigs fed 100 μg/kg had potassium levels similar to controls. Pigs fed 10 μg/kg of aflatoxin B_1 had significantly lower plasma glucose (119 vs. 140 mg/dl) when compared to pigs fed control diet. The average alkaline phosphatase value in the control pigs was 857 IU/L and was increased in pigs fed 100 and 200 μg/kg of aflatoxin B_1 by 5.7% and 10.6%, respectively.

In contrast, the effects of feeding aflatoxin during gestation on plasma parameters in pigs on the automatic feeding device are presented in Table 5. Plasma analysis revealed that sodium was depressed ($p<.05$) in pigs from sows fed 500 μg/kg of aflatoxin when compared to controls. Plasma carbon dioxide levels were different ($p<.05$) when comparing pigs from 500 and 750 μg/kg sows; however, neither of these values are different from control sow pigs. Alkaline phosphatase was increased ($p<.05$) in those pigs from sows fed 750 μg/kg of aflatoxin when compared to pigs from sows fed 500 μg/kg of

Table 5. The Analysis of Plasma Parameters for Pigs Reared in the Automatic Feeding Device Based on the Level of Dietary Aflatoxin Consumed by the Sow[1].

Parameters	Sow Treatment (μg/kg Aflatoxin)					
	Control (<20)		500		750	
Na, mmol/L	144.8	(1.5)[2,a]	139.2	(1.5)[a]	142.2	(1.2)
K, mmol/L	6.4	(0.2)	6.6	(0.2)	6.2	(0.1)
CO_2, mmol/L	25.4	(1.2)	22.9	(1.2)[a]	28.0	(1.0)[a]
Cl, mmol/L	106.1	(1.3)	103.7	(1.3)	103.9	(1.0)
Glucose, mg/dl	137.6	(5.7)	133.5	(5.7)	128.5	(4.5)
BUN, mg/dl	3.5	(0.3)	3.2	(0.3)	3.8	(0.2)
Ca, mg/dl	12.1	(0.3)	11.7	(0.3)	11.3	(0.2)
Protein, g/dl	4.5	(0.2)	4.3	(0.2)	4.2	(0.1)
Albumin, g/dl	2.5	(0.1)	2.5	(0.1)	2.3	(0.1)
Bilirubin, mg/dl	0.11	(0.02)	0.13	(0.02)	0.08	(0.02)
Alk. Phos.[3], IU/L	827.5	(72.1)*	802.7	(71.8)[a]	1011.5	(57.5)[a]
SGOT[4], mμ/ml	46.1	(4.6)	39.0	(4.5)	43.4	(3.6)

[1]The data presented for each parameter are an average of twelve blood samples in each sow treatment.

[2]Standard error.

[3]Alkaline Phosphatase.

[4]Serum glutamate-oxaloacetate transaminase.

[a]Means with the same superscript are significantly different, ($p<.05$).

*Approaching significant difference from 750 μg/kg sow treatment.

aflatoxin. This higher level is approaching significance ($p<.056$) when compared to pigs from control sows.

When twenty-two of these pigs were continued on either a dry or liquid milk based diets (Table 6), the pigs fed aflatoxin B_1 showed a reduced performance compared to pigs fed control diet. This effect was observed regardless of whether the liquid of dry diet was fed. Plasma retinol and alpha-tocopherol were also reduced even though statistical significance was not reached.

In nursery pigs fed corn-soybean meal diets, feed to gain ratios did not differ (data not shown). Pigs fed 100 μg/kg of aflatoxin gained similarly to control pigs (300 g/day) while those fed 300 μg/kg gained 10% less. However, pigs that consumed diets containing 500 μg/kg of aflatoxin had significantly lower 49 day weights (14 vs. 12 kg) and average daily gains (300 vs. 250 g/day) when compared to those pigs fed a control diet. Also, the plasma levels of retinol and alpha-tocopherol were significantly reduced as the level of aflatoxin increased in the diet (Table 7).

Table 6. The Effects of Aflatoxin on Nursery Pigs Fed Dry and Liquid Milk Based Diets[1].

	Dietary Aflatoxin B_1 (μg/kg)			
	Dry		Liquid	
	Control	1000	Control	1000
28-day weight, kg	4.93 (0.12)[2]	5.00 (0.12)	5.13 (0.20)	4.63 (0.15)
52-day weight, kg	14.58 (0.36)	13.21 (0.36)	15.65 (0.54)[a]	13.59 (0.44)[a]
ADG, kg	0.40 (0.10)	0.34 (0.10)	0.44 (0.14)	0.37 (0.12)
F/G	1.44 (0.28)	1.32 (0.28)	1.41 (0.41)	1.29 (0.34)

[1]A 24% protein diet consisting of nonfat dry milk (Bessiere and Co.), fat (HO-MILC, Merrick Foods), dextrose, mineral premix, vitamin premix, dicalcium phosphate, and choline (Calbiochem). The vitamins provided per kg of diet are 11,000 IU vitamin A; 1,100 IU vitamin D; 55 mg vitamin E; 10 mg vitamin K; 2.6 mg thiamine; 6 mg riboflavin; 44 mg niacin; 26 mg pantothenic acid; 44 μg vitamin B_{12}; 3 mg vitamin B_6; 0.2 mg biotin; 1.2 mg folic acid; and 1,100 mg choline. The mineral premix provided in percent of the diet copper, 0.0027; iron, 0.068; iodine (as iodized salt, 0.01% KI), 0.0088; manganese, 0.0091; selenium, 0.0001; and zinc, 0.045.

[2]Standard error.

[a]Means with the same superscript were significantly different, ($p<.05$).

Table 7. The Effects of Aflatoxin on Plasma Retinol and Alpha-Tocopherol in Nursery Swine[1].

	Treatment[2]			
	Control (<20)	100	300	500
Retinol[3]	42.52 (3.12)[4,a]	34.04 (2.82)[*,b]	31.01 (2.61)[a]	112.71 (2.43)[a,b]
Tocoph.[5]	184.00 (18.05)[a]	146.58 (16.80)[b]	128.95 (15.10)[a]	84.96 (14.1)[a,b]
No. of pigs	10	8	10	12

[1]Pigs were bled at 49 days of age.

[2]Micrograms of aflatoxin per kg of feed.

[3]Micrograms of retinol per 100 ml.

[4]Standard error.

[5]Micrograms of alpha-tocopherol per 100 ml.

[a,b]Means in the same column with the same superscript are significantly different, ($p<.05$).

[*]Approaching significance compared to controls, ($p<.054$).

Table 8. The Effects of Sow Treatment on Nursery Pig Growth.

	Sow Treatment (μg/kg Aflatoxin)		
Criteria	Control (<20)	500	750
No. of Pigs	28	38	35
21-day weight, kg	6.34 (0.24)[1,a,b]	4.99 (0.18)[a]	5.36 (0.22)[b]
49-day weight, kg	15.03 (0.54)[a,b]	12.52 (0.42)[a]	12.85 (0.50)[b]
Daily gain, g	310.0 (10)[a,b]	270.0 (10)[a]	270.0 (10)[b]

[1]Standard error.

[a,b]Means with the same superscript are significantly different, ($p<.05$).

When the data from the nursery study (corn-soybean meal diets) were analyzed considering the prior dietary exposure of the sows regardless of the pig's dietary aflatoxin exposure, effects emerged (Table 8). The 21-day weaning weights were depressed ($p<.05$). The average daily gains and average 49-day weights were also depressed ($p<.05$) regardless of the dietary aflatoxin concentrations to which the pigs were exposed.

Finishing Studies

Pigs fed aflatoxin to 100 kg body weight weighed less and gained less ($p<.05$) at 159 days of age than pigs fed control diet (Table 9). When comparing pigs from sows fed 500 μg/kg of aflatoxin with pigs from sows fed 750 μg/kg of aflatoxin (both groups fed 500 μg/kg), growth up to 49 days of age was similar. However, as these pigs increased in age and weight, the pigs from sows fed 500 μg/kg of aflatoxin grew slightly better at 159 days of age than the pigs from sows fed 750 μg/kg of aflatoxin. The effects of aflatoxin on growth were not reflected in the plasma values for retinol since no differences were observed. Alpha-tocopherol was not detected in these plasma samples.

Table 9. The Effects of Aflatoxin on Finishing Swine.

	Dietary Aflatoxin, μg/kg (Sow Treatment, μg/kg)		
Criteria	Control (Control)	500 (500)	500 (750)
No. of Animals	13	10	9
49-day weight, kg	16.40 (0.70)[1,a,b]	12.97 (0.80)[a]	12.97 (0.85)[b]
159-day weight, kg	97.61 (1.95)[a,b]	91.76 (2.22)[a]	88.93 (2.35)[b]
ADG, kg	0.74 (0.15)*	0.72 (0.17)	0.69 (0.18)*
F/G	3.02 (0.06)[a,b]	2.76 (0.07)[a]	2.72 (0.07)[b]

[1]Standard error.

[a,b]Numbers with the same superscript are significantly different, ($p<.05$).

*Approaching significance, ($p<.05$).

DISCUSSION

Baby pigs sucking sows fed 750 μg/kg of dietary aflatoxin consistently showed a reduced rate of growth. These observations were in agreement with data reported by Armbrecht et al., (1972), but in contrast to data of Hintz et al. (1967) who found that pigs sucking sows fed aflatoxin did not have a slower growth rate. This could be explained by the higher levels of aflatoxin used in the present study and that of Armbrecht et al. (1972). The present study utilized diets containing 14% crude protein while those of Hintz et al. (1967) contained 19-20% crude protein. Since the toxicity of aflatoxin appears to be reduced as the level of protein increases in the diet (Madhaven and Gopalan, 1968), this could also explain the conflicting results.

Our study and that of Armbrecht et al. (1972) have shown that aflatoxin does cross the placental membranes since aflatoxins were found in stillborn pigs. In the automatic feeding device where any affects of sow milking ability, variation in the amount of aflatoxin excreted, and nipple competition are eliminated, pigs from sows fed 750 μg/kg of aflatoxin had significantly reduced 24-day weights and average daily gains when compared with pigs from control sows. After 21 days away from the sow, the level of alkaline phosphatase which is reported to be elevated in pigs fed aflatoxin (Keyl et al., 1970) was significantly ($p<.05$) increased in pigs from sows fed 750 μg/kg of aflatoxin. These data suggested that aflatoxin when fed to the gestating sow has an effect on the newborn pig resulting in a negative growth response and altered plasma enzymes.

Aflatoxin has been clearly demonstrated to impair acquired immunity in swine by Cysewski et al. (1978) and in the turkey by Pier and Heddleston, (1970). Although immunological factors were not measured in this study, the immunosuppressive actions of aflatoxin may explain the increased mortality observed in the farrowing studies across sow treatments.

When pigs were reared with the aid of the automatic feeding device, reduction ($p<.05$) in growth was observed when pigs were fed 100 and 200 μg/kg of aflatoxin B_1. This reduction was similar to those pigs which had sucked sows fed aflatoxin. The level of aflatoxin M_1 found in sow milk (750 μg/kg) ranged from 0.01 to 3.90 μg/kg and 200 μg/kg of aflatoxin B_1 was required to give approximately the same reduction in growth. It is well known that aflatoxin B_1 is more toxic than aflatoxin M_1. Perhaps more aflatoxin M_1 was present in the sow milk than could be detected by the method used in this experiment since aflatoxin M_1 has been shown to bind to the casein molecule (Brackett and Marth, 1982). Or, perhaps aflatoxin B_1, in the liquid diet, was bound to casein in a manner similar to that of aflatoxin M_1, therefore reducing its toxic action.

The reduced nursery pig performance observed is in agreement with results obtained by Duthrie et al. (1966), but in contrast to data reported by Monegue et al. (1977). The differences in results could be explained by the source of aflatoxin used. Monegue et al. (1977) used toxin produced on rice culture whereas Duthrie et al. (1966) and the present study used naturally-contaminated feedstuffs.

The gestation and lactation of sows fed aflatoxin had depressive effects on baby pig performance. This effect persisted throughout the nursery study, and when aflatoxin-contaminated feeding was continued, this effect was seen in finishing swine.

Plasma retinol was reduced as the level of aflatoxin increased in the diet in the nursery study (corn-soybean meal diets) and in nursery pigs fed

milk based diets. Aflatoxin has been reported to lower liver retinol in pigs (Gumbman and Williams, 1969) and Keyl et al. (1970) reported that reduced liver retinol was accompanied by lower serum retinol levels. Lowered plasma retinol was not observed in the finishing swine suggesting that 500 μg/kg of dietary aflatoxin will not alter plasma retinol in market weight swine.

Plasma alpha-tocopherol was also lowered in the nursery pigs fed corn-soybean meal diets. To our knowledge, there are no reports on the interaction of aflatoxin and alpha-tocopherol. Aflatoxin has been reported to alter lipid metabolism (Smith and Hamilton, 1970) resulting in fatty livers. Since alpha-tocopherol is associated with body fat, perhaps more of this vitamin is being retained in the liver (fat) so that less is circulating in the plasma.

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An Epidemiological Study of the Association Between Delayed Estrus in Swine and Low Levels of Aflatoxin B_1 in Naturally Contaminated Feed

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INTRODUCTION

Mycotoxins are secondary metabolites of fungi found as contaminants of feed grains. Some mycotoxins, such as aflatoxin B_1 (AFB_1), zearalenone (ZE), deoxynivalenol, ochratoxin A, and T-2 toxin, are toxic to animals when ingested, inhaled, and when topically applied. Many of the toxins affecting animal production are produced by fungal species from three genera: Fusarium, Aspergillus, and Penicillium. These fungi are ubiquitous and under suitable environmental conditions will grow and produce toxic metabolites in feedstuffs and foods.

Swine of all ages, especially pigs and pregnant sows, are susceptible to the toxic effects of AFB_1 (Armbrecht et al., 1972; Hintz et al., 1967; Hoerr and D'Andrea, 1983). Clinical signs of acute exposure include anorexia, depression, lethargy, rough hair coat, icterus, severe hepatic pathology, hemorrhagic diarrhea, convulsions, and death (Armbrecht et al., 1972; Pier et al., 1977). More subtle indications of aflatoxicosis, such as increased susceptibility to infections, reproductive failures, and poor performance have been observed in swine chronically exposed to low levels (<100 μg/kg) of AFB_1. Whereas reproductive problems were not observed in pregnant sows exposed to 200 - 1100 μg/kg aflatoxins in their diets (Armbrecht et al., 1972; Hintz et al., 1967), McKnight et al. (1989) observed reduced growth rates in pigs born to sows consuming 500 and 750 μg/kg dietary AFB_1. Abortions and weak calves also occurred in gestating cows fed AFB_1-contaminated rations (Guthrie, 1979). There are also species differences in response to aflatoxin exposure (Edds, 1973; Newberne, 1973).

Clinical signs of reproductive failure in swine, such as anestrus, vaginal prolapse, rectal prolapse, pseudopregnancy, and reduced litter size have been associated with the ingestion of ZE. Both AFB_1 and ZE are common in feedstuffs on farms in the Southeastern United States (W.M. Hagler, Jr., unpublished).

The impact of low levels of mycotoxins, in particular those frequently found in grain, either alone or in combination, on production in swine under field conditions has not been fully investigated. The purpose of the study reported here was to evaluate subtle production problems associated with chronic low level exposure of sows to dietary aflatoxin. Specifically, days to return to estrus following weaning was used as a measure of the influence of AFB_1 on reproductive performance.

MATERIALS AND METHODS

Subject Selection

Swine confinement farms in North Carolina with similar husbandry and management levels were selected for study. These were divided into two groups: one with a suspected mycotoxin problem (high risk) and the other without a suspected problem (low risk). To be included as a high risk farm, large variability in health and production performance was necessary. After classification, five farms from each group were randomly selected for study. Animals on all farms consisted of crossbreed sows. A hand mating system was used and either commercial feed or feed mixed on-farm was fed.

Sampling of Feeds

Feed samples were collected from farrowing house feeders on the day before, or the day of weaning. Pooled subsamples, ca. 500 g of feed from each feeder in the farrowing group, constituted one sample. One sample was obtained per farm. Samples were placed in paper bags, labeled, and stored at 4°C until analyzed (3 days maximum). Samples obtained from the feeders during this time period were considered representative.

Dates of weaning, sow identification numbers, and condition of feeders were also recorded. Feeders were graded as good (frequent cleaning of feed, little or no feed buildup), fair (sometimes clean feeder, sometimes buildup), or poor (lots of old feed in feeder).

Mycotoxin Analysis

Samples were milled to pass a 1-mm screen, extracted, and analyzed for AFB_1 and ZE (Hutchins and Hagler, 1983; Swanson et al., 1984). Briefly, AFB_1 was determined by high performance liquid chromatography after conversion to the more intensely fluorescent hemiacetal. Zearalenone was determined by thin layer chromatography after extraction, lead acetate precipitation of lipids, and partitioning of ZE into toluene.

Statistical Analysis

For statistical analysis, a 2 x 2 contingency Chi-square design was employed (Mausner and Bahn, 1974). Previous studies indicated that >50 μg/kg AFB_1 in the feed resulted in aflatoxin M_1 milk residues in both cattle and swine (Stubblefield et al., 1983; Trucksess et al., 1983). Fifty μg/kg was chosen, a priori, to indicate high or low toxin levels for purposes of statistical analysis. Postweaning return to estrus, was defined as the number of days from weaning to breeding, with weaning day being day 1. The swine industry standard is to have sows return to estrus within 10 days of weaning. Therefore, we chose >10 days and <10 days for Chi-square analysis. All farms were visited 10 days postweaning. The only subject exclusions for this study were culled sows, because they were not bred. Univariate analysis (Chi-square, Student's t test) was used to determine any association between >50 μg/kg AFB_1 and delayed postweaning return to estrus.

RESULTS

One hundred thirty-one sows from the 10 farms selected were included in this study. The feed AFB_1 concentrations ranged from 2.2 μg/kg to 146 μg/kg with a mean of 44.7 μg/kg (Table 1). No ZE was found.

Table 1. Feed Aflatoxin Concentrations by Farm, Feeder Condition, and Date.

Farm	Feeder Condition	Date Weaned	Aflatoxin B_1 μg/kg
1	Good	5/17/86	2.2
2	Good	5/17/86	2.4
3	Good	5/25/86	8.2
4	Fair	5/25/86	15.0
5	Good	5/21/86	25.0
6	Good	5/21/86	36.0
7	Good	5/21/86	47.0
8	Good	5/17/86	70.0
9	Fair	5/17/86	95.0
10	Fair	5/17/86	146.0

The Chi-square analysis examining association of >50 μg/kg AFB_1 with delayed return to estrus is shown in Table 2. The data indicated an association between AFB_1 concentrations of >50 μg/kg in the feed and delayed return to estrus.

An estimated relative risk of ca. 3 (12 x 80/27 x 12 = 2.96) was also calculated (Mausner and Bahn, 1974). This result suggested sows consuming >50 μg/kg of AFB_1 in feed are 3 times more likely to have a delayed return to estrus than are sows consuming <50 μg/kg AFB_1. In addition, there was a difference between the feed AFB_1 mean of the group that returned to estrus in >10 days (58.8 μg/kg) and that of the group returning to estrus in <10 days (43.4 μg/kg) [$t_\infty = 5.13$, $p<0.001$].

No significant differences between the other variables were found. However, there was a trend that "good" feeders were associated with lower AFB_1 levels (Fisher's exact p = 0.06).

Table 2. Chi-square Analysis Indicating Association Between Dietary Aflatoxin B_1 and Delayed Return to Estrus in Sows.[1]

Return to Estrus	Aflatoxin B_1		Total Sows
	>50 μg/kg (No. sows)	<50 μg/kg (No. sows)	
>10 days	12	12	24
<10 days	27	80	107
Totals	39	92	131

[1]Chi-square $_{(1)} = 4.63$; p value = 0.003.

DISCUSSION

It would appear from this cross-sectional field study, that low level AFB_1 contamination was associated with delayed return to estrus. Several factors were not considered in this study: parity, duration of lactation, nutrition, boar exposure, and environmental factors. However, the farms were selected such that differences in management practices and facilities were minimal. Such factors should minimize differences resulting from mycotoxin ingestion. Assuming that randomization limited the influence of these factors, the results do not appear due to association of AFB_1 with other factor(s).

In addition, it was assumed that all sows in each farrowing group consumed the level of AFB_1 detected in the feeders, and that these levels were consumed over some period of time. These assumptions may be incorrect because aflatoxin is often found in "hot spots" in feed, and some of the feed bins were emptied weekly. However, this would argue against finding a significant difference. Therefore, in spite of these design factors, purposely handled to minimize the possibility of finding differences, significant associations were observed.

Postweaning return to estrus is a practical marker for reproductive performance of sows. Sow performance is also based on the number of pigs weaned/sow/year and litter weight at weaning (Pier et al., 1977). The longer it takes to breed postweaning, the lower the litters/sow/year and subsequently the fewer the pigs weaned/sow/year will be. Therefore, a major obstacle to achieving optimal reproductive performance on some swine farms could be due to the subtle effects of chronic AFB_1 ingestion. This study points out the need for further research in this area.

SUMMARY

Aflatoxin B_1 and zearalenone are known to cause productivity problems in swine. A field study of 10 commercial hog farms was conducted to determine the levels of aflatoxin and zearalenone in sow feed and to detect any association with postweaning return to estrus. No zearalenone was found in the feed. Incidence of detectable aflatoxin was 100%. Concentrations of aflatoxin ranged from 2.2 - 146 μg/kg with a mean of 44.7 μg/kg. A significant association ($p = 0.003$) was found between >50 μg/kg aflatoxin and delayed estrus (>10 days post-weaning). Results suggested that sows on a commercial swine farm with >50 μg/kg aflatoxin in sow feed has a greater chance of having delayed (>10 days) postweaning return to estrus.

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Subacute Toxicity of Cyclopiazonic Acid in Broiler Chicks Fed Normal and High Zinc Diets

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INTRODUCTION

Cyclopiazonic acid (CPA) was first isolated and identified as a toxic metabolite of Penicillium cyclopium Westling (Holzapfel, 1968). Corn and peanuts may be naturally contaminated with both CPA and aflatoxin B_1 (Gallagher et al., 1978; Lansden and Davidson, 1983). Cyclopiazonic acid was relatively toxic in most animal studies (Purchase, 1971; Dorner et al., 1983; Lomax et al., 1984; Morrissey et al., 1984; Morrissey et al., 1985; Nuehring et al., 1985; Cullen et al., 1988; Wilson et al., 1989) and the 72 hr oral LD_{50} of CPA in day-old broiler chicks was 12 mg/kg body weight (BW) (Wilson et al., 1989).

Cyclopiazonic acid is an indole tetramic acid (M.W. = 336.1) with the ability to chelate metal cations (Gallagher et al., 1978; Holzapfel, 1968). Another known chelator, ethylenediaminetetraacetate (EDTA), was shown to cause ulceration of the alimentary tract in dogs (Ahrens and Aronson, 1971). These lesions were associated with depletion of circulating zinc caused by infusion of EDTA. Similar alimentary tract lesions were described in dogs dosed orally with CPA (Nuehring et al., 1985).

The subacute toxicity of CPA has been partially described in broiler chickens (Dorner et al., 1983). Dorner et al. (1983) added CPA to feed at concentrations of 10, 50, and 100 mg/kg and fed ad libitum for 7 weeks.

There was a dose related decrease in weight gain and feed conversion. Lesions found in chickens consuming feed containing 100 mg/kg CPA consisted of proventricular ulceration and hyperplasia, necrosis of splenic parenchyma, and focal hepatocellular necrosis (Dorner et al., 1983).

Chickens appear to be very sensitive to the toxic effects of CPA. There is little information on the systems affected during subacute exposure to CPA in chickens. Therefore, the objectives of this study were: 1) to determine some of the effects of subacute exposure to CPA in broiler chickens; 2) to define effects of subacute exposure on various blood parameters in chickens; and 3) to determine if excess dietary zinc has a protective effect in chickens to subacute CPA exposure.

MATERIALS AND METHODS

Subacute Effects of Cyclopiazonic Acid

Day-old broiler chicks (Arbor Acre x Arbor Acre) were sexed and randomly assigned to 4 groups, each group consisting of either 10 male or 10 female chicks. Groups were dosed by gavage with 0, 1, 2, or 4 mg CPA/kg BW in corn oil. Cyclopiazonic acid was dissolved in corn oil as described by Wilson et al. (1989). The groups of chicks were randomly assigned to individual pens in Petersime brood units and doses (based on the average weight of chicks per pen) were administered daily for 23 days by oral gavage. Water and a standard broiler starter (23% crude protein) were available ad libitum. Weight gain, feed consumption, and water consumption were measured daily.

The chickens were bled via cardiac puncture on day 24, killed by cervical dislocation, and organs were weighed and tissues fixed for histologic examination. Hematocrits (packed cell volumes) for each bird were determined in heparinized microhematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA). Plasma sodium and plasma potassium were determined with a Nova 1 auto-analyzer (Nova Biomedical, Waltman, Massachusetts). The percent of plasma protein was determined using biuret protein analysis. Plasma glucose was analyzed using an automated glucose analyzer (Yellow Springs Instrument Co., Inc., Yellow Springs, OH) that utilized membrane-immobilized glucose oxidase. Plasma zinc was calculated using an atomic absorption spectrophotometer (International Laboratories, Model, IL 357).

Statistical analysis of the data was performed using an analysis of variance with levels of CPA and sex as the main effects. The general linear models procedure and Duncan's multiple range test were used to determine differences between means (SAS, 1982).

Subacute Effects of Cyclopiazonic Acid and Zinc

A 2x2x2 factorial arrangement of groups was used to study the effect of CPA and zinc on broiler chicks. The factors consisted of: the sex of chicks, the CPA dose (0 or 4 mg CPA/kg BW), and the diet type (standard broiler starter or broiler starter with 2000 mg/kg zinc [added as zinc oxide] per kg diet). Day-old broiler chicks (Arbor Acre x Arbor Acre) were sexed and randomly assigned to the dose and diet groups, each group consisting of either 10 male or 10 female chicks. The doses of CPA in corn oil (based on the average weight of chicks per pen) were administered daily for 22 days by oral gavage. Water and feed were available ad libitum. Weight gain, food consumption, and water consumption were measured daily.

The chickens were bled via cardiac puncture on day 22, killed by cervical dislocation, and organs were weighed and tissues taken for histologic examination. Hematocrits and plasma parameters including sodium, potassium, protein, and zinc were determined as described above.

Statistical analysis of the data was performed using a factorial analysis of variance with CPA, diet, and sex as the main effects. The general linear models procedure and Duncan's multiple range test were used to determine interactions and differences of treatment main effects (SAS, 1982).

RESULTS

Subacute Effects of Cyclopiazonic Acid

The subacute effects of CPA on organ weights in broiler chickens are shown in Table 1. There was no overall sex effect or interaction. Heart weight (%BW) was significantly lower ($p<.05$) in males and females receiving CPA. A significant increase ($p<.05$) in liver and pancreas weights (%BW) was seen in males and females at higher doses of CPA. Kidney weight (%BW) in females given 4 mg CPA/kg BW was significantly increased ($p<.05$), and kidneys in high dosed males tended to be larger. Spleen weights were not affected by CPA.

The subacute effects of CPA on blood and plasma parameters in broiler chickens are shown in Table 2. There was no overall sex effect or interaction. Hematocrit and plasma potassium were significantly reduced ($p<.05$) in CPA-treated males and females. Cyclopiazonic acid did not affect a change in sodium as compared to male and female controls. Plasma protein was significantly lower ($p<.05$) in females given 2 mg CPA/kg BW, and plasma protein in treated males tended to decrease. Glucose was significantly higher in males and females in the 1 and 4 mg/kg BW CPA group. There was a dose related decrease in plasma zinc in females, but not in males.

Water consumption was not altered by CPA. However, CPA did lower food consumption and weight gain in both males and females ($p<.05$) [data not shown]. Some mortality occurred during the 23 day study. Three male and three female chicks in the 4 mg/kg BW group died before the end of the experiment; one male chick in the 2 mg/kg BW group died early.

Table 1. The Subacute Effects of CPA on Organ Weights in Broiler Chicks.

	CPA			
Tissue	0 mg/kg BW	1 mg/kg BW	2 mg/kg BW	4 mg/kg BW
		Male[1] (G%BW)		
Heart	0.74 (0.033)[2]	0.62 (0.028)[a]	0.60 (0.024)[a]	0.63 (0.049)[a]
Liver	2.92 (0.136)	2.53 (0.122)	2.92 (0.162)	3.29 (0.089)[a]
Pancreas	0.32 (0.016)	0.31 (0.016)	0.38 (0.029)[a]	0.37 (0.018)[a]
Kidney	0.89 (0.075)	0.88 (0.047)	0.88 (0.030)	0.96 (0.027)
Spleen	0.14 (0.006)	0.11 (0.008)	0.12 (0.016)	0.10 (0.007)
		Female[3] (G%BW)		
Heart	0.77 (0.069)	0.60 (0.036)[a]	0.59 (0.022)[a]	0.59 (0.018)[a]
Liver	2.71 (0.083)	2.83 (0.136)	3.27 (0.308)[a]	4.20 (0.248)[b]
Pancreas	0.35 (0.028)	0.36 (0.024)	0.39 (0.015)[a]	0.42 (0.032)[a]
Kidney	0.92 (0.118)	0.85 (0.049)	0.97 (0.044)	1.14 (0.073)[a]
Spleen	0.12 (0.009)	0.11 (0.011)	0.13 (0.016)	0.13 (0.010)

[1] n=10 for 0 and 1 mg CPA/kg BW; n=9 for 2 mg CPA/kg BW; n=7 for 4 mg CPA/kg BW.

[2] Standard error of the mean.

[3] n=10 for 0, 1, and 2 mg CPA/kg BW; n=7 for 4 mg CPA/kg BW.

[a,b]Means in a row followed by different superscripts are significantly different ($p<.05$).

Subacute Effects of Cyclopiazonic Acid and Zinc

The subacute effects of CPA and dietary zinc on organ weights are shown in Table 3. There were no overall interactive effects. Cyclopiazonic acid significantly lowered ($p<.05$) heart weight (%BW) in male chicks fed normal diets. Liver and pancreas weights were increased in males and females treated with CPA. Both excess dietary zinc and CPA significantly lowered bursa weight (%BW) in males and females. Neither cyclopiazonic acid nor zinc affected kidney or spleen weights.

Table 2. The Subacute Effects of CPA on Blood and Plasma Parameters in Broiler Chicks.

Parameter	CPA							
	0 mg/kg BW		1 mg/kg BW		2 mg/kg BW		4 mg/kg BW	
				Male[1]				
Hematocrit (%)	30.6	(0.52)[2]	29.5	(0.22)	28.0	(0.66)[a]	25.5	(0.44)[b]
Potassium (mM)	5.39	(0.17)	5.10	(0.20)	4.73	(0.18)[a]	4.91	(0.18)[a]
Sodium (mM)	160.1	(2.54)	159.9	(2.76)	159.6	(1.67)	161.6	(1.42)
Protein (%)	3.11	(0.07)	3.05	(0.10)	2.72	(0.07)	2.91	(0.08)
Glucose (mg/dl)	253	(6.71)	272	(2.4)[a]	246	(8.34)	274	(2.84)[a]
Zinc (mg/dl)	124	(9.26)	131	(5.06)	109	(6.80)	122	(7.70)
				Female[3]				
Hematocrit (%)	30.2	(0.56)	29.9	(0.52)	27.0	(0.51)[a]	26.8	(0.95)[a]
Potassium (mM)	5.36	(0.15)	5.40	(0.16)	4.63	(0.17)[a]	5.04	(0.36)
Sodium (mM)	156.1	(2.46)	158.0	(2.11)	164.3	(3.09)	158.4	(2.24)
Protein (%)	3.14	(0.07)	3.64	(0.08)	2.72	(0.07)[a]	3.01	(0.17)
Glucose (mg/dl)	256	(6.07)	271	(7.2)[a]	232	(4.00)	267	(8.43)[a]
Zinc (mg/dl)	117	(6.75)	108	(5.61)	107	(9.77)	97	(14.10)[a]

[1] n=10 for 0 and 1 mg CPA/kg BW; n=9 for 2 mg CPA/kg BW; n=7 for 4 mg CPA/kg BW.

[2] Standard error of the mean.

[3] n=10 for 0, 1, and 2 mg CPA/kg BW; n=7 for 4 mg CPA/kg BW.

[a,b]Means in a row followed by different superscripts are significantly different ($p<.05$).

Table 4 summarizes the subacute effects of CPA and dietary zinc on blood and plasma parameters in broiler chicks. There were no overall interaction effects on these parameters. High dietary zinc increased hematocrit with and without CPA in males. In females, hematocrit was increased by CPA in chicks fed normal broiler starter. Cyclopiazonic acid decreased plasma sodium in males and females fed normal broiler starter. Both zinc and CPA caused an increase in plasma potassium and protein in females. However, CPA decreased plasma protein in male chicks fed normal broiler starter. Plasma zinc was significantly lower ($p<.05$) in CPA treated males and females on normal diets. Birds fed high zinc diets had increased levels of plasma zinc.

CPA significantly decreased ($p<.05$) food consumption and weight gain in both males and females. No significant zinc effects on feed consumption and weight gain were observed (data not shown).

Table 3. The Subacute Effects of CPA and Dietary Zinc (2000 mg/kg Zinc) on Organ Weights in Broiler Chicks.

	CPA			
	0 mg/kg BW		4 mg/kg BW	
	Diet (Zinc)			
Tissue	Normal	High	Normal	High
	Male[1] (G%BW)			
Heart	0.73 (0.050)[2]	0.76 (0.070)	0.63 (0.036)[a]	0.67 (0.044)
Liver	3.09 (0.172)	3.17 (0.178)	3.76 (0.256)[a]	3.79 (0.284)[a]
Pancreas	0.36 (0.016)	0.36 (0.012)	0.38 (0.032)	0.42 (0.034)[a]
Kidney	0.64 (0.072)	0.68 (0.097)	0.82 (0.188)	0.58 (0.076)
Spleen	0.14 (0.013)	0.11 (0.007)	0.23 (0.097)	0.12 (0.009)
Bursa	0.36 (0.031)	0.30 (0.023)[a]	0.27 (0.034)[a]	0.29 (0.017)[a]
	Female[3] (G%BW)			
Heart	0.70 (0.038)	0.61 (0.025)	0.68 (0.049)	0.62 (0.065)
Liver	3.53 (0.146)	3.35 (0.154)	4.18 (0.294)[a]	4.56 (0.209)[a]
Pancreas	0.40 (0.021)	0.40 (0.029)	0.46 (0.044)[a]	0.46 (0.022)[a]
Kidney	0.68 (0.099)	0.67 (0.094)	0.80 (0.124)	0.74 (0.089)
Spleen	0.14 (0.009)	0.10 (0.008)	0.13 (0.012)	0.27 (0.119)
Bursa	0.36 (0.020)	0.29 (0.028)[a]	0.27 (0.021)[a]	0.26 (0.022)[a]

[1] n=10 for normal and high zinc diets given 0 mg CPA/kg BW; n=9 for normal and high zinc diets given 4 mg CPA/kg BW.

[2] Standard error of the mean.

[3] n=10 for normal and high zinc diets given 0 mg CPA/kg BW; n=8 for normal diet given 4 mg CPA/kg BW; n=9 for high zinc diet given 4 mg CPA/kg BW.

[a] Means in a row followed by different superscripts are significantly different ($p<.05$).

DISCUSSION

Because the calculations of doses were based on the average weight of chicks per pen, chicks smaller than the average pen weight were administered higher levels of CPA on a body weight basis; larger chicks actually received less CPA on a body weight basis than other chicks in the dose group. These effects were magnified with time. Some smaller chicks in the 4 mg/kg BW groups died before the termination of the experiment probably because they received higher levels of CPA. This type of dosage scheme tended to minimize the CPA-induced effects; therefore, this study may have overlooked subtle effects of CPA.

Table 4. The Subacute Effects of CPA and Dietary Zinc (2000 mg/kg Zinc) on Blood and Plasma Parameters in Broiler Chicks.

	CPA			
	0 mg/kg BW		4 mg/kg BW	
	Diet (Zinc)			
Parameters	Normal	High	Normal	High
	Male[1]			
Hematocrit (%)	27.6 (0.49)[2]	30.7 (0.47)[a]	25.6 (1.15)	30.2 (0.94)[a]
Sodium (mM)	182.7 (6.07)	179.2 (3.26)	169.8 (4.73)[a]	174.6 (6.63)
Potassium (mM)	6.30 (0.26)	5.63 (0.23)[a]	6.20 (0.50)	6.11 (0.28)
Protein (%)	3.76 (0.15)	3.88 (0.12)	3.17 (0.40)[a]	3.71 (0.16)
Zinc (mg/dl)	212.0 (16.98)	325.6 (23.8)[c]	132.0 (22.63)[a]	261.7 (22.93)[b]
	Female[3]			
Hematocrit (%)	26.0 (0.89)	24.0 (2.59)	30.0 (1.69)[a]	27.4 (0.87)
Sodium (mM)	180.8 (4.47)	172.5 (2.0)[a]	171.4 (2.62)[a]	183.1 (7.13)
Potassium (mM)	5.62 (0.10)	6.04 (0.23)[a]	6.32 (0.20)[a]	6.47 (0.33)[a]
Protein (%)	3.34 (0.09)	3.88 (0.08)[a]	3.72 (0.12)[a]	3.77 (0.23)[a]
Zinc (mg/dl)	224.0 (17.65)	398.4 (26.6)[c]	183.0 (15.38)[a]	301.3 (21.68)[b]

[1] n=10 for normal and high zinc diets given 0 mg CPA/kg BW; n=9 for normal and high zinc diets given 4 mg CPA/kg BW.

[2] Standard error of the mean.

[3] n=10 for normal and high zinc diets given 0 mg CPA/kg BW; n=8 for normal diet given 4 mg CPA/kg BW; n=9 for high zinc diet given 4 mg CPA/kg BW.

[a,b,c]Means in a row followed by different superscripts are significantly different ($p<.05$).

Cyclopiazonic acid consistently decreased heart weight (%BW) in both studies. Cyclopiazonic acid has an affinity for skeletal muscle in the rat and chicken. Approximately 50% of CPA is distributed to skeletal muscle within 3 hr of IP or oral administration (Norred et al., 1985; Norred et al., 1988). The CPA-treated birds in this study exhibited elevated levels of plasma creatine kinase, an indicator of muscle injury (Cullen et al., 1988). These facts strengthen the importance of the association of CPA with muscle tissue, including cardiac muscle.

Cyclopiazonic acid caused enlargement of the liver, pancreas, and kidneys of chicks. Livers from treated birds appeared swollen and pale. Kidneys and pancreas sometimes appeared mottled.

Plasma protein decreased or tended to be lower in CPA-treated chicks in both studies. This may have been due to a direct effect of CPA on

transcription or translation; however, decreased plasma protein might be linked to muscle injury and repair mechanisms.

Histologic data from the chicks used in the subacute effects of cyclopiazonic acid study were reported by Cullen et al. (1988). These chicks developed lesions similar to birds given 100 mg/kg CPA in the feed by Dorner et al. (1983), but the lesions occurred with lower doses over a much shorter time period in birds given CPA daily by gavage. Skeletal muscle degeneration characterized by myofiber swelling or fragmentation occurred in the 4 mg/kg BW dose group (Cullen et al., 1988). Excess dietary zinc did not provide protection from the subacute toxicity of cyclopiazonic acid. Histologic examination of tissues revealed no dietary differences in frequency or severity of CPA-induced lesions in chicks.

Both CPA and the excess zinc caused a decrease in the size of the bursa of Fabricius. The reduction of bursal size is significant because of the compromise of the immune system of the growing chick. This would make the chick more susceptible to other stressors and decrease productivity (Glick, 1986).

Plasma zinc was lower in chicks given CPA. The mechanism of the reduction of plasma zinc may be the chelation and subsequent removal of zinc from circulation. However, another possibility is the binding of zinc by metallothionein in the liver.

Several parameters gave varying results or were not affected by subacute CPA toxicity. The mechanism of CPA toxicity appeared to be complex, interacting with many tissues and systems. A moderately toxic level of dietary zinc (2000 mg/kg) provided plasma levels of zinc in CPA-treated chicks approximately 50 mg/dl higher than plasma zinc levels in control birds consuming standard broiler starter diets. Although some CPA effects occurred only in chicks fed standard diets, CPA was toxic to the chicks fed high dietary zinc. Therefore, the chelation of zinc may not be important in the toxicity of CPA in broiler chicks.

SUMMARY

Cyclopiazonic acid (CPA) in corn oil was orally administered daily to 4 groups (0,1, 2, and 4 mg CPA/kg BW) of day-old broiler chicks for 23 days. There was a dose related increase ($p<.05$) in plasma glucose, and pancreas and liver weights (%BW) in each sex. Dose related decreases ($p<.05$) in hematocrit, plasma K^+, and heart weight (%BW) occurred in males and females. Plasma zinc and protein were lower ($p<.05$) in CPA-treated females. Groups of day-old broiler chicks were given normal or high zinc (2000 mg/kg) diets and 2 dosage levels daily of orally administered CPA (0 or 4 mg/kg BW). High dietary zinc had a protective effect ($p<.05$) on CPA-induced decreases

of plasma zinc. Some CPA effects only occurred in normal diet chicks, including increases ($p<.05$) in hematocrit, plasma K^+, and pancreas wt. (%BW) in females and decreases ($p<.05$) in heart wt. (%BW) and plasma protein in males. Feed consumption and weight gain were lower ($p<.05$) in birds treated with CPA in both studies. The chelation of zinc was not indicated as a major cause of CPA toxicity. The toxicity of CPA in broiler chicks was complex, involving many tissues and systems.

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Immunochemical Methods for Aflatoxins

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INTRODUCTION

Drought in 1988 caused a high incidence of aflatoxin contamination in corn samples in nine states including TX, OK, IA, IN, IL, SD, MD, WI and MN (Wall Street Journal, 1988a). In Illinois, 27% of 52 corn samples contained more than 20 ng aflatoxins/g (Wall Street Journal, 1988b). On October 4, 1988, the U.S. Food and Drug Administration (FDA) established enforcement levels for aflatoxins in corn used in feed (Rothschild, 1988). The levels were 100, 200 and 300 ng/g depending on the animal consuming the feed containing the aflatoxin-contaminated corn. FDA enforcement action could also be supported when corn containing in excess of 20 ng total aflatoxins/g was destined for food used by humans, for feed used by immature animals and by dairy animals, or if its destination was not known.

Simple, specific, sensitive (at levels of concern) immunochemical assays have become more popular and have been used to replace some of the traditional thin-layer chromatographic and high-performance liquid chromatographic (HPLC) methods; this has occurred because of the demand for analysis of large numbers of samples for aflatoxins in the field, in grain elevators and in laboratories without well-trained chemists. These methods can be used to quickly sort out negative samples and identify positive samples.

Presently there are three types of immunochemical methods: radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA) and immunoaffinity column assay. The formation of highly specific, multiple noncovalent bonds between the toxins and the immobilized antibody on a solid phase is the basic principle of the commonly used immunoassays for aflatoxins. RIA techniques have extremely high sensitivity and objectivity. However, RIA reagents may have short half-lives and the radioisotopes require special handling, including waste disposal and safety measures.

Therefore, the most common techniques today are those which utilize enzymes instead of radioisotopes as labels. The ELISA consists of a two-step process: first, the reaction between the enzyme-labeled antibody and the toxins, and then detection of the reaction product, using the enzyme-catalyzed reaction of the substrate with the enzyme-labeled antibody-toxin complex.

Many ELISA tests have been developed (Pestka, 1988; Chu, in press). Two commercial test kits, the Agri-screen and the Afla 20 Cup, have been collaboratively studied and approved by the Association of Official Analytical Chemists (AOAC) as official first action methods for screening for aflatoxins in several commodities (Park et al., 1989a, 1989b; Trucksess et al., 1989).

With the advent of monoclonal antibody techniques and the development of affinity column technology, antibodies were bound covalently to beaded agarose. The affinity column is used to bind the aflatoxins to the antibody-matrix. The column serves as a concentration tool for aflatoxins (Candlish et al., 1988; Groopman and Donahue, 1988). The only immunoaffinity column for aflatoxins manufactured in the United States has been evaluated and found to give results similar to those obtained by the CB method (Trucksess et al., 1990a in press). An AOAC/IUPAC (International Union for Pure and Applied Chemistry) collaborative study was conducted to validate the use of this immunoaffinity column for corn, peanuts and peanut butter (Trucksess et al., 1990b in press). In this study total aflatoxins were determined, after elution from the immunoaffinity column, by solution fluorometry with bromine, and individual aflatoxins were determined by HPLC post-column derivatization with iodine.

In the paper herein, we are primarily concerned with the criteria for the evaluation of commercial immunoassay kits and our collaborative studies of some of these kits.

CRITERIA FOR EVALUATION OF COMMERICAL IMMUNOASSAY KITS

In the past few years, a number of immunoassay kits for aflatoxins have become commercially available. A list of some of the manufacturers and the types of immunoassay kits is found in Table 1. This listing includes those for which our laboratory had received descriptive information and is not intended to be all-inclusive.

There are no formal standard criteria for evaluation of the kits. Several organizations have been actively engaged in developing evaluation guidelines (Pohland et al., 1989): AOAC, IUPAC, Environmental Protection Agency, and U.S. Department of Agriculture (USDA). Some of the criteria for the evaluation of immunoassays that have been used in our laboratory are listed in Table 2.

Table 1. Commercially Available Aflatoxin Immunoassay Kits.

Company Name and Country	Types
Cambridge Life (U.K.)	MTP[a]
May and Baker (U.K.)	MTP, AC[b]
Oxoid (U.K.)	AC
Thames Genelink (U.K.)	MTP
Environ. Diag. Sys. (U.S.A.)	Card
Idexx (U.S.A.)	MTP, Cup, Probe
Idetak, Inc. (U.S.A.)	MTP
Inter. Diag. Sys. (U.S.A.)	Cup
Vicam (U.S.A.)	AC
Neogen (U.S.A.)	MTP
Transia (France)	MTP
UBE (Japan)	MTP

[a]Microtiter plate

[b]Affinity column

Table 2. Criteria for Evaluation of Performance of Immunoassay Kits for Aflatoxin Analysis.

INFORMATION PROVIDED BY MANUFACTURERS

Sensitivity

Standard curve plotting absorbance versus concentration, indicating assay range, 50% inhibition concentration

Specificity

1. Cross-reactivity with other aflatoxins
2. Matrix interference

Avidity

Affinity constants and stability of antibody-antigen complex

Applicability

1. Commodity
2. Target level
3. Condition = temperature, humidity

Stability

1. Shelf life of reagents
2. Environmental (Heat, humidity)
3. Maximum repeated uses (some reagents require equilibration to room temperature before use)

Procedure

1. Sample Preparation
2. Extraction
3. Analysis

Quality Control

1. Lot to lot variation
2. Within lot variation

Cost and equipment

INFORMATION OBTAINED BY USERS

Precision

1. Within a plate or kit, different plate or kit
2. Different sample extract

Accuracy

Percentage recoveries or percentage false positive and false negative

Simplicity

1. Ease of use
2. Time required

IMMUNOASSAY STUDIES AND RESULTS

After carefully evaluating some of the kits using above criteria, we conducted four collaborative studies. The first collaborative study involved use of the Neogen Agri-Screen kit. Polyclonal antibodies specific to aflatoxin B_1 were coated onto plastic microtiter wells. This test provides either qualitative answers or semi quantitative results, depending on whether visual comparison or an ELISA "reader" was used to evaluate the color reaction. The study design, procedure and results are found in Tables 3 and Table 4 (Park, et al., 1989a).

Table 3. Neogen Agri-Screen Collaborative Study.

Collaborators: 14

Commodities: Corn, raw peanuts, roasted peanuts, whole cottonseed, cottonseed meal, mixed feed

Analyte and Level: Aflatoxin B_1, 15 and 50ng/g

Extraction: Weigh 5 g sample into 50 mL test tube with screw cap. Add 25 mL MeOH-H_2O (55 + 45) and 10 mL hexane, shake 1 minute. Filter, separate layers, and save lower layer

Immunoassay Procedures (9):

Set up mixing wells. Pipet 0.1 mL aflatoxin B_1 enzyme conjugate solution into two control wells and one well for each standard (15, and 50 ng/g) and each sample. Add 0.1 mL aflatoxin B_1 standards and sample extract to each well and mix. Set up antibody coated wells. Pipet 0.1 mL of solution from each mixing well to antibody coated well. Let stand for 5 minutes. Empty wells, and wash well 10 times with H_2O. After final wash, invert wells on paper towel and tap to remove excess H_2O. Add 0.1 mL substrate solution (mixture of ABTS and H_2O_2) to each well. Let stand 10 minutes. Add 0.1 mL color stopping solution.

Determination:

Visual: 15 ng/g standard: grey-green color 50 ng/g standard: pink color sample: if darker green than 15 ng/g standard then <15 ng/g if lighter green or pink then >15 ng/g.

ELISA reader: Use 405nm filter and calibrate on a well containing only substrate and color stopping solution. Then read standards, control and sample well.

Table 4. Results of Neogen Agri-Screen Collaborative Study-I.

Commodity	Level ng/g	Instrumental ng/g	Visual -	(% False) +
Corn	5	2.8 ± 2.6	-	15
	26	12.6 ± 8.1	36	-
Raw peanuts	6	21.8 ± 7.9	-	85
	20	21.8 ± 8.7	4	-
Roasted peanuts	3	4.1 ± 4.4	-	8
	28	16.5 ± 13.4	31	-
Cottonseed	36	35.2 ± 15.9	17	-
	85	41.0 ± 12.3	0	-
Cottonseed meal	6	8.1 ± 4.9	-	44
	31	18.6 ± 9.3	12	-
Mixed feed	4	5.9 ± 5.3	-	35
	14	15.9 ± 4.3	23	-

AOAC adopted the Agri-screen test kit official first action as a screening method for determining the presence (>15 ng/g) or absence (<15 ng/g) of aflatoxin, in cottonseed products and mixed feeds.

A second collaborative study of the Agri screen test kit was conducted. The study design and procedure were identical as the first study except tetramethylbenzidine was used instead of ABTS as substrate. (Park et al., 1989b). Results of this study are shown in Table 5.

Table 5. Results of Agri-Screen Collaborative Study - II.

Commodity	Level ng/g	Instrumental ng/g	Visual -	(% False) +
Corn	4.8	11.2	-	0
	31.0	56.0	0	-
Roasted peanuts	1.5	9.2	-	6
	39.0	70.2	0	-
Raw peanuts	10.8	125.0	-	13
	34.0	83.0	0	-

On the basis of these results the AOAC adopted the method as an official first action screening method for aflatoxin B_1 in corn and roasted peanuts at >20 ng/g.

A third collaborative study was recently completed for the Immuno Dot Screen Cup. In this system antibodies were covalently attached to a membrane and the membrane was contained in a cup like device. The method was design to quickly identify test sample containing <20 ng/g total B_1, B_2 and G_1 as negative in aflatoxins, and test sample containing >20 ng/g aflatoxins as positive. Visual judgement was used to interpret the results. The development of color indicated the test sample contained aflatoxins below the determination level, while no color development indicated the test sample contained aflatoxins at ≥20 ng/g. The joined AOAC/IUPAC collaborative study procedure and results are listed below in Table 6 and Table 7 (Trucksess, 1989).

Table 6. Immuno Dot Screen (IDS) Cup Collaborative Study.

Collaborators: 12

Commodities: Corn, raw peanuts, feed, cottonseed, peanut butter

Level: 30, 20, 10, 0, ng/g aflatoxins, B_1 : B_2 : G_1 = 10 : 1 : 3

Extraction: Add 100 mL MeOH-H_2O (8 + 2) to 50 g sample (except peanut butter) in a blender jar. Blend 3 min and filter. Prepare test extract by adding 400 uL dilution buffer to 200 uL filtrate. Peanut butter (50 g) is blended for 3 minutes with 100 mL hexane and 250 mL MeOH (55 + 45). Filter, and separate layers, then heat on steam bath for 3 minutes. Dilute 500 uL filtrate with 500 uL buffer to prepare test extract.

Immunoassay:

Apply three 200 uL peanut butter test extract to the cup with a one minute wait between each 200 uL aliquot. Apply two 150 uL other test extracts to the cup with a one minute wait between each addition and before proceeding to the next step. Apply 100 uL (2 drops) enzyme solution and wait 1 minute. Wash with 1.5 mL (30 drops) wash solution. Add 1.0 mL substrate solution. Wait one minute and observe disk for blue color development (negative) or no color development (positive).

Determination:

Negative: If the disk (center of the cup) turns light blue or darker, the test sample contains <20 ng/g total aflatoxins B_1, B_2 and G_1.

Positive: If no blue is observed in disk, the test sample contains ≥20 ng/g total aflatoxins B_1, B_2 and G_1.

Table 7. Immuno Dot Screen (IDS) Cup Collaborative Study.

Commodity	Level ng/g	%+	95% Conf. Int. Lower	Upper
Corn	NC	100	88	100
	30	92	73	99
	20	75	53	90
	10	33	16	55
	0	0	0	22
Raw Peanuts	NC	100	88	100
	30	96	78	100
	20	83	62	95
	10	54	33	75
	0	0	0	22
Poultry feed	30	83	62	95
	20	46	26	67
	10	4	0	21
	0	0	0	22
Cottonseed	60	96	79	100
	20	96	79	100
	10	58	37	100
	0	0	0	22
Peanut Butter	30	100	87	100
	20	91	72	99
	10	61	39	80
	0	0	0	24

All collaborators correctly identified naturally contaminated corn (101 ng/g) and raw peanut (69 ng/g) positive samples. No false positives were found for control samples containing <2 ng/g. The correct responses for spiked positive samples at 10, 20 and >30 ng/g levels were 52, 86 and 96% respectively. The method was rapid and simple and was adopted Official First Action by the AOAC as a screening procedure for aflatoxins at >20 ng/g in corn, cottonseed, peanuts and peanut butter and >30 ng/g in poultry feed.

The fourth collaborative study was for the Aflatest-P immunoaffinity column for aflatoxins in corn, peanuts and peanut butter. After the immunoaffinity column cleanup, the total aflatoxins were determined by solution fluorometry with bromine (SFB) and individual aflatoxins were determined by HPLC post column derivatization with iodine (PCD). An outline of the procedure is shown in Table 8 (Trucksess, et al. 1990b).

The results of the above study (Table 8) are tabulated on Table 9 and Table 10.

Table 8. Aflatest-P Immunoaffinity Column Collaborative Study.

Collaborators: 24, 12 collaborators used SFB, 9 used PCD, and 3 used both SFB and PCB.

Commodities: Corn, peanuts, peanut butter

Level: 30, 20, 10, 0, ng/g (B_1 : B_2 : G_1 : G_2 = 7 : 1 : 3 : 1)

Extraction:

Weigh 25 g sample and 5 g NaCl in a blender jar. Add 125 mL MeOH-H_2 (7 + 3) Blend 2 min. Filter and collect 15 mL. Add 30 mL, mix and filter.

Affinity column chromatography:

Add 15 mL second filtrate to column reservoir. Use syringe pump to push sample through column. Wash column with 2 x 10 mL H_2O. After washing pass 2-3 mL air through column. Add 1 mL HPLC grade methanol to reservoir. Pass methanol through column and collect in: (a) for quantitation by SFB use a 12 x 75 mm borosilicate test tube. (b) for quantitation by PCD, use 2 mL volumetric flask.

Quantitation:

By SFB: Calibrate fluorometer with blank and 20 ng/g standard. Add 1 mL 0.002% bromine in water to eluate. Mix and insert sample tube in fluorometer. Wait 60 second and record reading. This reading is equivalent to ng total aflatoxins/g sample.

(cont.)

Table 8. Continued.

By PCD

Column: 4.6 mm x 25 cm, 5 um C18
Mobile phase: H_2O-CH_3CN-MeOH (3 + 1 + 1)
Flow rate 1.0 mL/min.

Post column reagent: 0.05% iodine water
Reaction coil: 0.5 mm x 610 cm, 70° C.
Post column reagent flow rate: 0.3 mL/min.

Standard curve: Use mixture of B_1 : B_2 : G_1 : G_2 = 0.250 : 0.063 : 0.125 : 0.063. Use 3 x this concentration for the highest standard.

Sample: Inject 50 uL test extract. Identify each aflatoxin peak in sample by comparing retention with corresponding reference standards.
Determine quantity of each aflatoxin in test sample injected from corresponding standard curves. Calculate concentration of each aflatoxin in the sample.

Table 9. Immunoaffinity Column Collaborative Study (Solution Fluorometry with Bromine).

Commodity	Level ng/g	RSD_r	$_gSD^R$	Recovery %
Corn	NC	19.57	22.95	-
	30	16.57	20.01	105
	20	-	14.44	106
	10	-	33.09	124
	0	-	56.48	-
Peanut Butter	30	-	10.97	111
	20	-	20.55	107
	10	11.75	13.57	131
	0	30.45	36.51	-
Peanuts	30	-	13.44	97
	20	12.79	15.34	105
	10	-	27.49	115
	0	87.18	107.15	-

Table 10. Immunoaffinity Column Collaborative Study (LC Post Column Derivatization).

Commodity	Level ng/g	RSD_r	RSD_R	Recovery %
Corn	NC	21.44	21.48	-
	30	7.31	11.71	83
	20	-	4.71	83
	10	-	50.77	72
	0	-	115.55	-
Peanut Butter	30	-	21.74	78
	20	-	23.60	81
	10	16.95	29.60	90
	0	85.81	123.06	-
Peanuts	30	-	14.23	79
	20	5.13	15.97	80
	10	-	36.27	83
	0	230.55	268.53	-

In general, the recoveries for SFB were over 100% of the amount added to the samples. The higher recoveries could be due to fluorescent interferences in the sample matrix. For PCD the averages of the analytical results for total aflatoxins for the 10, 20 and 30 ng/g samples ranged from 81 to 84% of the amount added. The recoveries of B_1 were greater than 80%. The recoveries of B_2, G_1 and G_2 varied considerably because of the small amounts added to the samples.

Based on this collaborative study, the affinity column chromatographic method was recommended by the Mycotoxin General Referee, Dr. Peter Scott, for adoption as an official AOAC method.

SUMMARY

A number of immunoassay kits for aflatoxins are now available. Four of these kits were evaluated by collaborative studies. They were found to be suitable for aflatoxin analysis in certain commodities. These test kits and the procedures using them are continuing to be improved based to a large degree on the experience of these studies. While criteria to be used for the evaluation of these kits for aflatoxins are not yet standardized, this issue is under active consideration by USDA and FDA. These immunochemical methods have been demonstrated to be very simple and in most cases sensitive and specific at the levels of concern. They are already assuming an important role in aflatoxin monitoring programs.

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Wall Street Journal, October 6, 1988b.

A Proposed Immunoassay for T-2 Toxin

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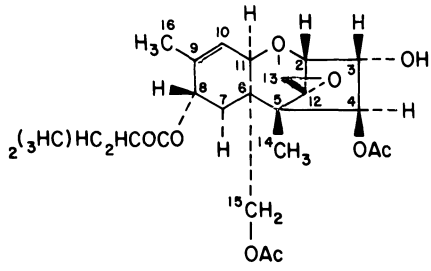
INTRODUCTION

Overview of Trichothecenes

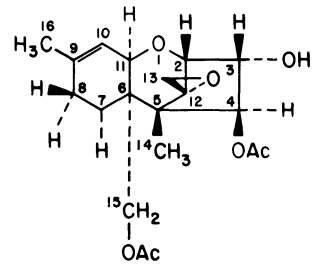
Previously (Uneo, 1983; Dashek et al., 1985; Lacey, 1985) reviewed the evidence regarding the alleged utilization of certain trichothecenes as biological warfare agents and the biological effects and chemistry of the trichothecenes as well as the fungi which produce them. The agricultural commodities which have been reported to contain trichothecenes have been summarized (Dashek et al., 1985). The structures of certain trichothecenes which are known to occur in the commodities are depicted in Figure 1. Of these, the preeminent trichothecene appears to be T-2 toxin.

Currently Available Screening Methods for T-2 Toxin

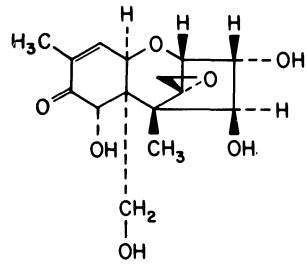
These include: thin layer (TLC), high performance liquid (HPLC) and gas liquid (GLC) chromatographies as well as combined gas chromatography-mass spectrometry (GC-MS). When TLC is used, colors can be developed within those plate areas containing trichothecenes by spraying with 20% aluminum chloride, 20% sulfuric acid, 0.32% phenylhydrazine, anisaldehyde or 10% sulfuric acid and 10% glacial acetic acid in methanol followed by heating at 110°C. Trichothecene levels can be quantified by either visual comparison of these regions with those containing standards upon the sample plate or by densitometry. Because



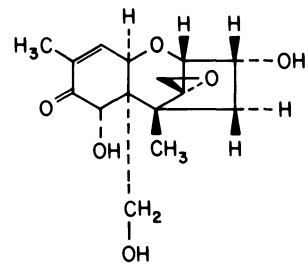
T-2 TOXIN



4,15-DIACETOXYSCIRPENOL (DAS)



NIVALENOL



DEOXYNIVALENOL (DON)

Figure 1. Structures of Diacetoxyscirpenol, T-2 Toxin, Deoxynivalenol and Nivalenol.

the epoxy ring of trichothecenes reacts with 4-(p-nitrobenzyl)-pyridine (Takitani et al., 1979) or with nicotinamide and 2-acetyl-pyridine (Sano et al., 1982), the products can be directly quantified upon TLC plates either visually or densitometrically.

Gas liquid chromatography is an effective method for quantifying trichothecene derivatives (trimethylsilyl ethers or heptafluorobutyrates) in solutions. These derivatives can be detected by flame ionization detectors (FID) (Collins and Rosen, 1979; Kuroda et al., 1979; Mirocha et al., 1976; Yamamoto, 1975), electron capture detectors (Kamimura et al., 1981; Romer et al., 1978, Yamamoto, 1975) or mass spectrometry with or without selected ion monitoring (SIM) (Collins and Rosen, 1979; Illus et al., 1981).

Another chromatographic procedure which could be modified for the resolution of trichothecenes is the high pressure liquid chromatography method of Pons and Franz (1977).

Each of these procedures involves sophisticated, expensive instrumentation and some possess the disadvantages of lack of specificity and sensitivity. Here, is proposed the development of a thin layer immunoassay (TIA) for the quantitation and detection of T-2 toxin.

Historical Development and Value of Thin Layer Immunoassays

Several immunological methods have been described for the detection of either unnatural or foreign materials (xenobiotics) within biological samples. These include: a free radical assay technique (FRAT) (Leute et al., 1971, 1972), radioimmunoassay (RIA) (Spector and Parker, 1970; Castro and Malkus, 1977), enzyme immunoassay (EI) (Rubenstein et al., 1972) and hemagglutination inhibition (HI) (Adler and Liu, 1971). Although these procedures are exquisitely sensitive, each suffers from one or more of the following disadvantages: an appreciable initial equipment expense; a requirement for both specialized reagents and materials; a relatively short shelf-life for some of the reagents; a need for both special safety requirements and/or operating licenses, and complicated multi-step procedures and difficulties in performing these in a non-laboratory setting.

In contrast, TIA offers an attractive alternative to these immunoassay methods since it is not dependent upon either specialized equipment or radionuclides; is relatively rapid; and is capable of detecting pg quantities of xenobiotics. Although TIA has been developed and applied to a variety of chemical tests (Elwing et al., 1976, 1977a,b;

Jeansson et al., 1979), there is no report in the literature regarding the utilization of TIA as a procedure for both detecting and quantifying either T-2 toxin or other mycotoxins. This may stem from difficulties in producing anti-T-2 toxin (Chu et al., 1979).

The TIA depends upon the fact that many antigens (Ag) and antibodies (Ab) will bind as monolayers of molecules to hydrophobic surfaces such as plastics. Usually, these proteins retain their immunological properties subsequent to the binding thereby permitting the performance of Ag-Ab reactions (hereafter termed the primary reactions) in that area of the monolayer to which the antiserum was applied.

One of the major advantages of TIA is that the occurrence of the primary reaction can be detected via simple visualization procedures carried out in situ (Elwing et al., 1976). The simplest of these procedures is a vapor condensation upon surface (VCS) method which is based upon the fact that the area of the Ag-Ab reaction is more hydrophilic than the antigen monolayer since the area contains more protein than the monolayer surrounding it. Therefore, when a plastic's surface upon which an Ag-Ab reaction has occurred is exposed to water vapor, the reaction is visualized with ease since it is more ""wettable"" than the surrounding monolayer. Consequently, the water droplets which condense in the region of Ag-Ab reactions are larger than those which condense upon the portion of the plate which contains only Ag monolayer. This difference in condensation pattern, while often visible to the unaided eye, is more accurately observed at low power (10-30 x) of a light microscope. Several visualization methods are available other than VCS. Two of these, serological reinforcement and mixed hemabsorption, have been reported to be approximately 10-300 times more sensitive than the VCS method. Serological reinforcement involves the incubation of another antiserum upon the plastic surface subsequent to the primary reaction. This antiserum is directed against the original species from which the initial antiserum was produced (the primary reaction). The application of this antiserum results in the addition of another layer of protein to the area of the Ag-Ab reaction, thereby enhancing the hydrophilicity of the area. Visualization of the serologically reinforced primary reaction is accomplished by the VCS method.

The mixed hemabsorption visualization technique is performed by using red blood cells (RBCs) sensitized with a sub-agglutinating amount of antibody against a RBC determinant (an anti-receptor may be employed). The antiserum used for the sensitization of the RBC is prepared in the

same species as was the anti-serum employed for the primary reactions. The sensitized RBCs are added to the plastic's surface following the serological reinforcement and attach to the plastic in the area of the primary reaction since the antiserum employed for the serological reinforcement reacts with both the antiserum used in the primary reaction and the anti-serum which sensitized the RBCs.

The major disadvantage of TIA is that quantitation, although possible (Elwing et al., 1976), is both time-consuming and requires an additional incubation at 60°C. However, we propose to develop a semi-quantitative method for the detection of xenobiotics, which could be performed as rapidly as the qualitative assay, by using more than one dilution of inhibited antiserum.

The development of TIAs for T-2 toxin promises rapid, sensitive, inexpensive procedures for both the detection and quantitation of the toxin within biological samples. In addition, its potential as a portable procedure renders it applicable to on-site testing of air, water, food, animals, plants, inorganic materials, as well as human blood, and urine.

Some obstacles reported above have been overcome in part by the very recent availability of both T-2 toxin conjugated to BSA protein and rabbit polyclonal antibody conjugated to T-2 toxin. The Department of Forensic Sciences at George Washington University has played a major role in the adaptation of the technique to analytical problems in both forensic serology and toxicology. Lappas and his associates have developed the first TIAs for the detection of a hapten, e.g., opiates in both urine (Fredenburg and Lappas, 1982) and blood (Shughart and Lappas, 1983), as well as fetal hemoglobin in blood (Whitehead et al., 1983), and the identification of human blood stains (Lappas, 1980; Lappas and Fredenburg, 1981).

OBJECTIVES

It is hypothesized that a thin layer immunoassay for T-2 can be developed and that the assay can be applied to both the detection and quantitation of the toxin. To accomplish these tasks, it is necessary to develop both rapid and reproducible TIAs for the detection and quantitation of T-2 toxin; to test the application value of the TIA for both detecting and quantitating suspected T-2 within blood, organ extracts, urine, and vegetation; and to originate and test modifications of the TIA for field use by non-technical personnel.

Additional objectives include: obtaining cytotoxicity data; production of monoclonal antibodies to replace polyclonal antibody to T-2 toxin; and use of monoclonal antibodies to detect and localize T-2 toxin in both cells and tissues.

METHODS

The methods which could be employed for the development of a TIA for T-2 toxin are as follows:

Objective 1: Development of TIAs for Both the Detection and Quantitation of T-2 Toxin, and the Assembling and Toxicity Data for the Production of Monoclonal Antibodies to T-2 Toxin

As previously mentioned, both T-2-BSA and anti-T-2 toxin have been prepared. The rationale and problem in the production of both the conjugate and antibody have been described in "Protection Against Trichothecene Mycotoxins" (National Academy Sciences, 1983).

The analytical method to be developed for the detection of T-2 toxin could be an inhibition method of the TIA using the VCS method of visualization. Much of the antisera required for this research is available. The thrust of the research should involve an evaluation of both the feasibility and sensitivity of such an analytical procedure. The method to be developed consists of four steps: the formation of monolayers with T-2-BSA conjugates; inhibition of anti-T-2; incubation of the inhibited anti-T-2 upon the monolayers; and visualization of the T-2-anti-T-2 complexes by means of the VCS method. This method was selected because it is the simplest of the three previously described visualization methods. The use of serological reinforcement and hemabsorption methods of visualization would require evaluation, should the VCS method not provide sufficiently sensitive results. The research for objective 1 might center upon the determination of optimal conditions of monolayer formation and antisera dilution and the establishment of quantitative methods of analysis.

Monolayer formation and antisera titer determination using the VCS method: Monolayers could be formed using T-2-BSA conjugates. The anti-T-2 protein conjugates should be quantitated by spectrophotometry. The parameters of monolayer formation to be evaluated include: a) concentration of the T-2-BSA complexes to be employed; b) volume of the T-2-BSA complexes to be utilized; c) type of plastic surface (polystyrene

or polyvinyl chloride); and d) length of incubation (1, 5 or 15 mins.). Antisera solutions (3 µl) ranging from undiluted to dilutions of 1:64 would be applied to each of the formed monolayers and incubated at room temperature for 1 to 60 mins.

Visualization of Ag-Ab reactions will be accomplished by the VCS method. The results of these experiments will be evaluated so that the combination of antiserum dilutions, antiserum incubation time, and conditions of monolayer formation which yield the most unequivocal results might be selected. Wherever possible, the selected parameters will be designed around short incubation times and the smallest quantity of reagents.

Cross Reactivity. Experiments should be performed to determine whether trichothecenes other than T-2 toxin, e.g., diascetoxyscirpenol, deoxynivalenol, and nivalenol, interfere with the TIA's for T-2 toxin. Concentration ranges from the ng through the mg range should be employed. Control and crude plant, as well as animal extracts, require "spiking" with known quantities and agricultural grain samples from State, Federal (USDA) and certified laboratories. Some of these relate to collaborative studies often undertaken by organizations such as the Association of Official Analytical Chemists and American Oil Chemists Society.

Quantitation. The degree of antiserum inhibition should be related to the number of xenobiotics present within the sample. Therefore, samples containing elevated concentrations of the xenobiotic will be able to inhibit both dilute and concentrated antisera, whereas samples containing low concentrations will be able to inhibit only dilute antisera. Since the product (water droplets) of the VCS method can be photographed, a quantitative method involving the relationship of droplet size to antigen concentration is feasible.

Blind Investigations and Controls. A blind investigation will be conducted in order to evaluate the reliability of the TIA. Urine, blood and vegetation samples containing T-2 and/or its metabolites at several different concentrations will be both extracted and coded. After this, and once the VCS has been perfected, triplet upon triplet tests will be undertaken. They will include more blind samples and controls as well as control and tests completed by consultants and assistants for each type of sample.

Toxicity of T-2 Toxin in Mice Previous to the Development of a Monoclonal Antibody to T-2

The feasibility of immunizing mice with T-2-BSA conjugate is

suggested by the findings of Scheifer (1982) who reported that mice are capable of overcoming T-2-induced (210 ppm) cutaneous, gastrointestinal tract and radiometric lesions in both lymphoid and hematopoietic organs. The methods of Scheifer could be modified as necessary. To do this, at least 20 groups of 15 mice and controls should be used for concentration gradient doses. Preliminary groups to be tested include carriers starting with water, DMF, alcohol and DMSO.

In Vivo Toxicity/Immunization

To establish optimal conditions for immunization and to provide additional in vivo toxicity data, 10 groups of experimental and control Balb/c mice should be inoculated via I.P., I.M. or I.V. immunization routes. We anticipate that there will be a necessity for 30 mice initially in each of these groups. The dosage levels and route of inoculation of both T-2 toxin and T-2-BSA conjugate should be determined based upon data of pilot investigations with CD-1 mice. Those mice which survive the acute insult could be subjected to another immunization/inoculation via the same route with a dose calculated to be approximately the LD_{10} for that species at four weeks after the initial injection. The LD_{10} should be determined by procedures based upon 10 groups of mice having 10 animals per group and receiving a range of dilutions causing 0 to 100% deaths in 30 days. The exact concentration may be read from a lethality curve generated from the above test.

Three to four weeks subsequent to the second injection survivors could be sacrificed for their sera. Dilutions (1:100) of the sera might be used to evaluate competitive inhibition of the TIA which could be developed using rabbit polyclonal antibodies directed against T-2 toxin. Specific competition of the TIA could be taken as evidence of obtaining an antibody to T-2 toxin which can be quantified. These data may provide the basis for both the immunization route and its schedule for Balb/c mice in order to develop monoclonal antibodies to T-2 toxin. Selected (young adult male) Balb/c mice (three groups of 10) could be injected with T-2 toxin at the LD_{50} and then sacrificed during the acute state (i.e., 6-24 hrs when the animals should become lethargic as based upon CD-1 mice in our neonate studies). To determine pathology, all major visceral and thoracic organs and tissues should be prepared and examined by light microscopy.

Additionally, certain tissues might be examined for T-2 localization by immunocytochemical methods to be developed at a later stage of the investigations described below.

Objective 2: The Application Value of the TIA for Detecting and Quantitating Suspected Aflatoxin or T-2 Within Biological Samples and to Develop Monoclonal Antibodies to T-2 Toxin

Ten extracts each of blood, urine and vegetation (rice and tree leaves) should be spiked with known amounts (previously quantified by capillary chromatography or T-2 (Sigma, St. Louis, MO) standards. Both the blood and urine will be collected from non-exposed human subjects (healthy laboratory personnel following standard approved format by established and filed procedures). In the case of vegetation, 10 samples of 25 species could be painted with T-2 within a solvent, and T-2 applied directly onto the surface of the leaves and stems.

The recommended T-2 extraction procedures are methanol-water (Kamimura et al., 1981; Romer et al., 1978), methanol in water (Yamamoto, 1975), and ethyl acetate (Illus et al., 1981; Kuroda et al., 1972; Scott, 1982). Because the specificity of an Ag-Ab reaction is high, crude extracts allow for rapid assay. However, a variety of purification procedures, e.g., ion-exchange (Yamamoto, 1975; Mirocha et al., 1976; Kamimura et al., 1981) chromatography and/or ferric chloride (Collins and Rosen, 1979) are required.

The data obtained from the TIAs for T-2 should be compared with those derived from gas capillary chromatography (Cohen and Lapointe, 1980) for T-2. These comparisons will involve the use of both t-tests and an analysis of variance (Snedecor and Cochran, 1972) to establish the validity of TIA results relative to the standard techniques that are presently available.

T-2 Toxin Monoclonal Antibody Production/Analysis

Monoclonal antibodies to T-2 toxin as a substitute for the rabbit polyclonal antibodies should be generated. In addition, these antibodies would also provide the specific probe (with appropriate label) for immunocytochemical localization of T-2-BSA conjugate. Four weeks after the final immunization (second injection) mice would be given a final booster. Two days subsequent to the booster the mice are sacrificed; the spleens removed; and a suspension of spleen cells subjected to fusion with nonsecretor mouse myeloma cells P_3 653 Ag_8 using PEG 4000. Hybridomas are selected in HAT medium during the first week of culture. Supernatants from viable hybridomas are collected and analyzed following 10 days of culture by competitive inhibition of the TIA assay which uses polyclonal rabbit antiserum to T-2.

All cultures exhibiting competition should be expanded and cloned

using supernatants from RAW264.7 cells to provide B-cell growth factors in the culture medium. The expanded and cloned hybridoma cells could be assayed for production of monoclonal antibodies to T-2 toxin. All monoclonals to T-2 require serotyping and some of the cells frozen for reserve. High titer pools of antibodies are produced by growth in the peritoneum of Balb/c mice for employment in both TIA and immunocytochemistry.

Once monoclonal antibodies to T-2 toxin have been obtained, an evaluation of the possibility of substituting T-2 monoclonal antibody for rabbit polyclonal antibodies in the TIA to achieve enhanced sensitivity and specificity should be undertaken. Also, if multiple monoclonals to T-2 are obtained, mixtures of the monoclonals can be evaluated in a "co-operative" immunoassay if desired to accelerate assay sensitivity as has been demonstrated recently for human chorionic gonadotropin (HCG).

Objective 3: Originate and Test a Modification of TIAs Which Could Permit Their Use in the Field by Non-Technical Personnel and Employ Anti-T-2 Monoclonal Antibodies in Immunocytochemical Localization of Each in Mouse Tissues and Cultured Cell

Because of the ease with which the TIA can be performed and the compactness of the equipment, several field kits for agricultural use suggest themselves, e.g., pre-packaged mini-microtiter plates. A kit composed of aliquots of lyophilized but easily reconstituted anti-T-2 could be constructed. Alternatively, it is possible to pre-coat surfaces (wells) with T-2 BSA. Personnel would carry out an antiserum inhibition and then mix the antiserum at various dilutions of crude extract of material to be tested thereby performing the TIA except for the visualization. A portable device which would generate a water vapor mist at 60°C could be provided for the visualization (sterno can and coffee pot or hot water steam).

Immunocytochemical Localization of T-2 Toxin in Both Cells and Tissue

Localization of T-2 toxin in tissues could be undertaken by using an immunoperoxidase technique which we recently developed. Tissues of Balb/c mice will be prepared for microscopic examination, sectioned and finally stained for antigen (T-2 toxin). The tissue and immunoperoxidase procedure has been described. These same techniques could be used for T-2 toxin localization using a second antibody (rabbit-anti-mouse) conjugate with the enzyme.

In addition, human diploid cells, IMR-90, could be examined after exposure in culture to determine the ultimate sensitivity and specificity of the immunoperoxidase assay and help provide a time-course of the fate or disposal of the antigen in individual cells. Chlorox (Yang, 1972) and acetone (Castegnaro et al., 1981) will be available for both clean-up and detoxification as needed. Detoxified T-2 toxin should be removed for incineration. The red bag system for incineration is recommended.

SUMMARY

The trichothecenes are biologically-active fungal metabolites which can be synthesized both in vitro and in vivo by various Fusarium, Trichothecium, Trichoderma, Cephalosporium, Myrothecium, Stachybotrys, and possibly Dendrodochium species. Because trichothecenes occur in agricultural commodities, it is necessary to develop rapid, inexpensive, reliable, accurate and sensitive assays for the toxin. Here, we propose an immunological method for both the detection and quantification of T-2 toxin. This method involves the production of a monlayer of T-2 toxin within polyvinyl chloride microtiter wells. The subsequent steps include in sequence: a) washing and drying of the monolayer; b) incubation of the layer with bovine serum albumin (BSA); c) washing and drying of the BSA-toxin layer; d) incubation of the layer with anti-T-2 toxin; and e) washing and drying of the anti-T-2 toxin-BSA-toxin layer. Finally, the toxin-antitoxin complex is both visualized and quantified via a water vapor technique.

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Cytotoxicity of *Fusarium moniliforme* Metabolites

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INTRODUCTION

There is little doubt that the genus Fusarium constitutes one of the most important groups of toxigenic fungi. Many species of Fusarium produce an array of secondary metabolites which elicit dissimilar physiological and pharmacological responses in plants and animals. Many of these are mycotoxins. More than half of the 37 trichothecenes that have been isolated are elaborated by Fusarium species (Vesonder and Hesseltine, 1985). Fusarium moniliforme is one such member of this genus. It is a common, ubiquitous fungal pathogen to many plants, including corn (Zea mays); it causes corn stalk and ear rot diseases (Ayers et al., 1972). The range of secondary metabolites produced by F. moniliforme includes mycotoxins, antibiotics, phytotoxins and phytoalexins. Although the fungus is known to be internally seed borne in apparently healthy corn kernels (Foley, 1962; Marasas et al., 1979), it has been correlated with increased human esophageal cancer risk in China and Southern Africa (Marasas et al., 1984), and has been implicated as the causative agent of equine leucoencephalomalacia (ELEM) (Marasas et al., 1976). Both moniliforme (Cole, 1973) and Fusarin C (Wiebe, 1981) have been rejected as the causative agent of ELEM. Direct and unequivocal evidence linking a mycotoxin of F. moniliforme with ELEM has been shown by Marasas et al., 1988. Recently, a novel water soluble metabolite of F. moniliforme with cancer promoting activity was isolated and characterized (Bezuidenhout et al., 1988). This mycotoxin has been shown to be responsible for ELEM, but its involvement in the high incidence of esophageal cancer in humans in the Transkei region of South Africa is yet to be proven. Hence continued investigation into the production of toxic metabolites by F. moniliforme is warranted.

Bioassays are used for screening fungal extracts for the presence of known or unknown toxins. While chemical tests are generally regarded as being more sensitive, only known mycotoxins can be assayed

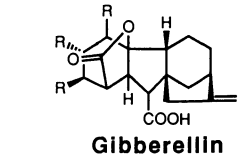
in this way. Consequently, many mycotoxins would go undetected if only chemical tests were relied upon. Bioassays are therefore absolutely essential during the preliminary stages of discovery and identification of new toxic fungal metabolites. Once a mycotoxin has been identified, chemical assays may be developed which would replace bioassays in the detection of the new toxin.

The toxicity of several Fusarium metabolites has been determined in Hep-2 cells (Robb and Norval, 1984), including the F. moniliforme metabolites moniliformin and zearalenone. The purpose of this study is to determine the effect of seven metabolites of F. moniliforme (Figure 1) on four mammalian cell lines; to assess the sensitivity of these cells for the detection of toxic metabolites of F. moniliforme.

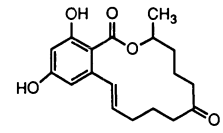
MATERIALS AND METHODS

Metabolites

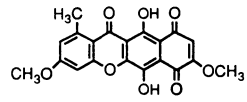
Seven metabolites of F. moniliforme were used including, moniliformin, zearalenone, fusaric acid, dihydroxy fusaric acid, fumonisin B_1, bikaverin and gibberellin. One milligram quantities were weighed and 1 ml of methanol added to each vial with the exception of bikaverin where a 50:50 mixture of methanol:methylene chloride was used as the solvent.



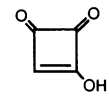
Gibberellin



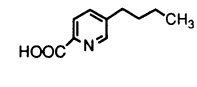
Zearalenone



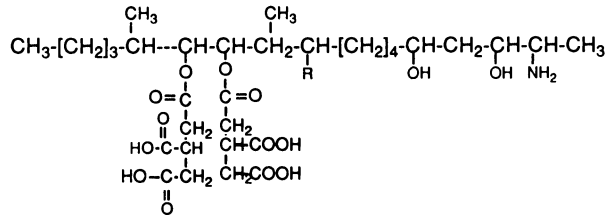
Bikaverin



Moniliformin



Fusaric acid



Fumonisin

B_1: R=OH
B_2: R=H

Figure 1. Fusarium moniliforme Metabolites.

Cell Culture

Baby hamster kidney cells (BHK), human epithelial cells, Hep-2 cells, African green monkey kidney cells (Vero), and McCoy mouse cells were obtained from The American Type Culture Collection in Rockville, MD for use in this study. The cells were grown in Earles minimum essential medium supplemented with inactivated newborn calf serum in 25 cm^3 tissue culture flasks and placed in an incubator at 37° ± 1°C with an atmosphere of 5% carbon dioxide. When the monolayer was formed, the cells were trypsinized and suspended in 1 ml medium and diluted with culture medium to provide a concentration of 1 x 10^5 cells/ml. A volume of 0.2 ml of this cell suspension was dispensed aseptically into sterile 96 well plates (Falcon) and put into a CO_2 incubator at 37°C where a monolayer formed overnight. One, 2, 3 and 4 µl volumes of the metabolites were inoculated into the monolayer culture of cells. T-2 toxin was used as the positive control and carrier solvent as the negative control. Wells with nothing added were also included in the study. The 96 well plates were reincubated after incubation at 37°C in a CO_2 incubator for 24 hours. The medium was then decanted and the cells fixed with absolute ethanol and stained with Giemsa stain. The well plates were then air dried and examined under an inverted microscope for evidence of cell death.

RESULTS AND DISCUSSION

Table 1 depicts the effect of the seven metabolites on the four cell lines used.

Moniliformin

Moniliformin was first isolated by Cole et al., 1973, from a strain of F. moniliforme isolated from corn seeds damaged by Southern leaf blight in the United States. This compound has been found to be highly toxic to one-day-old cockerels, LD_{50} 4.0 mg/Kg of body weight administered per os, and is phytotoxic to corn and tobacco (Cole, 1973). Because of its toxicity to chickens and its phytotoxicity it was considered important to determine its cytotoxic effects as well. Table 1 indicates that moniliformin does not exhibit any cytotoxic effects in this study up to a concentration of 4 µg.

Fusaric Acid

Fusaric acid is a well known phytotoxin (Gaumann, 1957), but is not generally regarded as a mycotoxin. However, in view of the fact that fusaric acid is a chelating agent, it has been implicated as a contributor to abnormal bone development in broiler chicks. Vero, McCoy and Hep-2 cells were not affected by 1, 2, 3 or 4 µg of Fusaric acid. Only the BHK cell showed signs of cell death at 3 & 4 µg concentrations. Dihydroxy fusaric acid was also effective against BHK cells but at a lower concentration of 2 µg.

Table 1. Levels of Detection of Metabolites in Monolayer Culture.

Mycotoxin	VERO score[a]	VERO vol[b] μl[c]	BHK score	BHK vol μl	McCoy score	McCoy vol μl	HEP-2 score	HEP-2 vol μl
Moniliformin	0	4	0	4	0	4	0	4
Fusaric acid	0	4	1	3	0	4	0	4
DH Fusaric acid	0	4	1	2	0	4	0	4
Zearalenone	3	2	1	2	1	4	1	4
Fumonisin	0	4	1	1	0	1	1	4
Bikaverin	1	3	1	1	1	2	1	1
Cibberellin	0	4	1	3	0	4	1	4
T-2 toxin	1	4	3	1	0	4	3	1
methanol	0	4	0	4	0	4	0	4
$CH_3OH:CH_2Cl_2$	0	4	1	4	0	4	1	4

[a]0 = no cell death; 4 = complete cell death; 2 & 3 = intermediate effect.
[b]Vol = volume added.
[c]1 μl contained 1 μg.

Zearalenone

The first report that F. moniliforme produced the estrogenic metabolite, zearalenone, was made by Mirocha et al., 1969. Zearalenone is the major toxin responsible for vulvovaginitis or the estrogenic syndrome in swine. The syndrome can be induced experimentally by administration of the purified toxin to swine and other animals. One to five ppm zearalenone in the feed of swine produces physiological effects. The cytotoxicity of zearalenone is shown in Table 1. Both Vero and BHK cell lines were effected by zearalenone at 2 μg. The McCoy and Hep-2 cell lines were also affected by 4 μg of zearalenone.

Gibberellins

The gibberellins were originally isolated as plant growth promoters from cultures of F. moniliforme by Yabula and Hayashi (1939) during a study of Bakanae disease of rice in Japan. Mirocha et al., 1974 reported estrogenic and uterotrophic effects of gibberellic acid to the possible involvement of this group of compounds to uterine hypertrophy caused by cultures of F. moniliforme in mice and rats.

Gibberellin demonstrated cytotoxic effects to both BHK and Hep-2 cells at 3 and 4 μg concentrations respectively. Vero and McCoy cells were not affected at the highest concentration added.

Fumonisin

Bezuidenhout et al., 1988, characterized a novel metabolite produced by F. moniliforme. This compound, called Fumonisin, has been shown to have cancer promoting activity (Gelderblom, 1988) and has been shown to induce ELEM in South African horses (Marasas et al., 1988). Table 1 indicates that BHK cells are most sensitive to Fumonosin at 1 μg concentration. Hep-2 cells were also found to be affected by Fumonisin B_1 but at a higher concentration than BHK cells indicating a higher sensitivity of BHK cell to this mycotoxin.

Bikaverin

Bikaverin is a wine red pigment with marked and specific antiprotozoa activity. It was first isolated from fermentation of Gibberella fujikurdi (=F. moniliforme) by Balan et al., 1971. The cytotoxic effects of Bikaverin have not been reported. In this study, all 4 cell lines were found to be sensitive to Bikaverin with BHK and Hep-2 cells being the most sensitive (Table 1).

SUMMARY

It is evident from this study that metabolites of F. moniliforme elicit varied responses in different cell lines. Moniliformin does not exert a cytotoxic effect on the cell lines used, probably indicating the need for metabolic activation before it could become toxic. The BHK cell line appears to be the most sensitive cell line for the detection of F. moniliforme metabolites. In the event a fungi such as Fusarium, Alternaria, Penicillium, or Aspergillus are suspected in a mycotoxin incidence and the metabolite responsible can not be detected by comparison with known compounds, the use of mammalian cell lines offers a sensitive, relatively inexpensive and rapid method of detecting toxic metabolites.

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Microorganisms Associated with Shelled Corn Stored in Above and Below Ground Storage Facilities

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INTRODUCTION

Underground storage of grain and dry edible beans is a management technique that has developed independently on every continent in the world. Several ancient cultures, including those that developed in what is now the U.S., used underground storage (Ambler, 1977). As long ago as 1000 B.C., some cultures used underground storage as their principal means of strategic national storage (Dunkel, 1985; Sterling et al., 1983). Both modern and ancient reports have claimed that insect infestation, fungal growth and taste acceptability are well-controlled in underground structures; some grains could be stored well for 10-12 years in such structures.

Several underground structures currently used in other parts of the world may have application for the grain storage situation in the midwest area of the U.S. In particular, the use of polyvinyl chloride sheets, bags, and liners has reduced the capital investment required while providing a natural insecticidal and fungicidal atmosphere. Underground structures have been used successfully in large-scale experiments in Brazil with edible beans and corn (Sartori, 1982); in Australia with wheat (Champ and McCabe, 1984); in Argentina with wheat (Republica Argentina, 1949); and in Israel with barley (Donahaye et al., 1967). Small scale experimental studies with pearl millet in the U.S. at Manhattan, Kansas, have shown some control of insects during storage through the use of underground facilities (Asanga and Mills, 1985).

The objective of this study was to compare the microbial flora of shelled corn stored in above and below ground metal storage structures and assess the potential for aflatoxin contamination.

MATERIALS AND METHODS

Site Selection

The site of the field storage trials was on the University of Minnesota campus in St. Paul, MN. The area is part of the Anoka sand plain; the soil at site of the trials is a sand and gravel soil type to a depth of 7.6 m. This work was part of a larger experiment described by Dunkel et al., (1987).

Storage Structure

The storage structures were cylindrical metal bins chosen because of similarity to the facilities that are currently used for on-farm storage of shelled corn. The bins were 2.26-m-high and 1.42-m-wide. The bins were originally manufactured as hog feeders and the feed holding compartment was modified to be a closed unit. All seams and bolts on the bin were sealed with waterproof acrylic sealant during the final assembly. The lid on each bin was a tight-fitting convex form with an overlap of 3 cm. The lids had an external clamp. In order to access the corn and remove samples, three apertures were made in each lid and plastic conduits were attached to the metal lids with epoxy. Just prior to bin filling a backhoe was used to create an earth opening approximately 0.2 m larger than the bin. The bin was lowered into the opening and filled with corn using a movable auger. When the grain was in place, the bin was closed and the same earth was replaced along the sides and top of the bin so that a 0.5-m-deep mound of earth covered the entire bin. When finished, the plastic conduits stood 0.5 m above the earth cover. These were neither insulated nor covered with earth during the experiment. The conduits were fitted with caps to prevent moisture from entering the bins.

Corn

The corn used was a yellow dent type with an initial moisture content of 15.6 ± 0.7%. Samples were taken after 36 months of storage in the bins at the termination of the experiment. Samples were taken from five locations in the bin, north side, east side, south side, west side and center. At each location samples were taken at five levels, surface, 18 inches (46 cm), 36 inches (91 cm), and 48 inches (123 cm) below the surface, and at the bottom of the bin. The moisture content was determined using the electronic Motomoco meter (Model 919). The samples were sieved with a 12/64 round metal sieve using the standard stroke method. The percentages of broken corn and foreign material were also determined, as was an insect count.

Microbiological Analyses

The microbial content of the corn was measured by three different techniques. First of all a portion of each sample was ground and serial

dilutions were made for total yeast and mold counts. These counts were done using the plate count technique on dichloran rose-bengal chloramphenicol agar (DRBC) described by King et al., (1979). In addition Aspergillus flavus counts were done using A. flavus-parasiticus agar (AFPA) as described by Pitt et al., (1983). The third method used was the direct plating of whole, surface sanitized corn kernels on four different media (Food and Drug Administration, 1984). The kernels were surface sanitized by soaking in 5% NaOCl solution for 1 min and rinsing three times with sterile distilled water. The kernels were placed directly on the agar surfaces in standard petri dishes, 10 kernels/plate. A total of 100 kernels were plated on each of four different media. The media used were potato dextrose agar (Difco) plus tetracycline (PDAT), dichloran rose-bengal chloramphenicol agar (DRBC) and the same two media plus 7.5% NaCl. The percentage of mold infected kernels per sample was determined.

Aflatoxin Analyses

Aflatoxins were extracted from samples using 80% methanol and filtered through fluted filter paper (Whatman #2). The filtered extracts were diluted three-fold and filtered again through Whatman 934-AH glass microfibre filters. Filtered extracts were then loaded onto affinity chromatography columns containing monoclonal antibodies specific for aflatoxin B_1, B_2, G_1 and G_2 (Aflatest, VICAM, Somerville, MA). The columns were washed with two 10 ml quantities of distilled H_2O and the aflatoxins were eluted from the columns using 1 ml of HPLC grade methanol. The purified aflatoxin samples were then analyzed in a fluorometer (Sequoia-Turner Model 450) calibrated to read out as ppb aflatoxin.

RESULTS

The moisture content of the corn increased from 1 to 2% during the storage period. There was no observed insect activity and no viable insects in corn stored below ground, however, insect activity was noted in the corn stored above ground. There was some visible mold on kernels at the surface of the corn and in the first layer.

Total mold counts were higher in the corn stored below ground (Table 1). In general, counts were highest at the surface of the grain and into the first layer of the corn to about 18 inches (46 cm). Samples taken at lower levels in the bin had lower mold counts. The underground bin was apparently vandalized when the caps on the sampling conduits were removed, allowing moisture to enter the bin. Total molds in corn stored above ground were lower and appeared to be of the same magnitude throughout all five

levels of the bin. For the most part, yeasts were not detected in the corn, however, samples that contained detectable yeasts tended to have lower mold counts. Two bottom samples (one each from above and below ground bins) that had no detectable mold, had yeast counts of 10^4 and 10^5 respectively.

Table 1. Total Mold Counts of Corn Stored in Below and Above Ground Metal Storage Structures.

Location in Bin	Level of Grain Sampled				
	Surface	18 inches (46 cm)	36 inches (91 cm)	48 inches (123 cm)	Bottom
			Below Ground		
North	2.6×10^6	6.6×10^5	5.4×10^4	2.5×10^3	9.7×10^2
East	1.5×10^{6a}	____[b]	2.2×10^5	4.7×10^2	N.D.[a,c]
South	1.9×10^6	4.2×10^5	7.9×10^4	1.8×10^4	1.3×10^3
West	3.4×10^5	2.5×10^6	3.2×10^5	2.5×10^4	1.1×10^3
Center	9.3×10^{2a}	8.1×10^5	5.8×10^4	____[b]	2.1×10^3
			Above Ground		
North	1.9×10^3	2.7×10^2	3.7×10^{2a}	7.3×10^{2a}	N.D.[a,c]
East	3.2×10^3	7.7×10^2	2.5×10^3	5.7×10^2	____[b]
South	2.0×10^3	4.3×10^3	3.0×10^2	1.0×10^3	2.7×10^{2a}
West	3.3×10^6	4.1×10^5	4.4×10^3	1.10×10^{3a}	____[b]
Center	1.3×10^3	6.3×10^2	2.2×10^5	2.0×10^4	7.0×10^2

[a]Samples also had high yeast counts.
[b]Data not available.
[c]ND-None Detected.

The percent of mold infected kernels was found to be higher when determined on PDAT and DRBC plus salt than on the media without salt. These media gave very similar results, so only the results from PDAT plus salt are given. The percent infected kernels was higher in corn stored below ground than above ground (Tables 2 and 3). With corn stored below ground the highest percentages of infection were found at the surface of the grain, though all layers had high percents of infection. With corn stored above ground the percent infection was high at the surface for two sample areas, but low in the other three areas. With corn stored above ground the percentages of infected kernels throughout the bulk were somewhat higher than at the surface.

Table 2. Comparison of Percent Mold Infected Kernels, Aspergillus flavus Counts and Aflatoxin Content (μg/kg) in Corn Stored in a Below Ground Metal Storage Structure.

Location in Bin	Level of Grain Sampled				
	Surface	18 inches (46 cm)	36 inches (91 cm)	48 inches (123 cm)	Bottom
North					
Infected	82%	75%	82%	72%	51%
A. flavus	20	5	10	ND	100
Aflatoxin	ND[a]	21	ND	ND	1
East					
Infected	87%	—[b]	38%	78%	68%
A. flavus	65	—	15	100	ND
Aflatoxin	5	—	ND	ND	ND
South					
Infected	85%	68%	61%	49%	80%
A. flavus	15	25	ND	100	100
Aflatoxin	ND	2	3	ND	ND
West					
Infected	76%	83%	69%	67%	82%
A. flavus	35	30	10	50	100
Aflatoxin	ND	ND	3	ND	ND
Center					
Infected	39%	86%	88%	—[b]	64%
A. flavus	15	240	ND	—	100
Aflatoxin	ND	ND	2	—	ND

[a]ND - None detected.
[b]Data not available.

Table 3. Comparison of Percent Mold Infected Kernels, Aspergillus flavus Counts and Aflatoxin Content (μg/kg) in Corn Stored in a Above Ground Metal Storage Structure.

Location in Bin	Level of Grain Sampled				
	Surface	18 inches (46 cm)	36 inches (91 cm)	48 inches (123 cm)	Bottom
North					
Infected	15%	53%	68%	71%	19%
A. flavus	20	ND[a]	15	ND	ND
Aflatoxin	ND	5	1	ND	2
East					
Infected	7%	84%	68%	38%	—[b]
A. flavus	50	15	45	ND	—
Aflatoxin	2	ND	10	10	—
South					
Infected	63%	58%	61%	75%	73%
A. flavus	140	180	5	35	ND
Aflatoxin	ND	ND	ND	ND	5
West					
Infected	96%	86%	74%	54%	—[b]
A. flavus	100,000	540	15	100	—
Aflatoxin	166	5	2	ND	—
Center					
Infected	8%	41%	96%	61%	70%
A. flavus	ND	ND	15,000	120	5
Aflatoxin	ND	4	1	ND	1

[a]ND - None detected.
[b]Data not available.

Aspergillus flavus counts tended to be very low in samples from both bins (Tables 2 and 3). Except for two samples in the above ground bin, most samples of corn had A. flavus counts well below 10^3/g, with most being 200/g or less. An inherent difficulty in doing the A. flavus counts was the need to sufficiently dilute samples to prevent overgrowth of the AFPA plates by non-A. flavus molds.

Aflatoxins were found in some of the corn samples, but for the most part levels were low (Tables 2 and 3). Only two samples had aflatoxin levels above 20 μg/kg. Fifty-four percent of the samples (27/50) contained no detectable levels of aflatoxin. The sensitivity of the test is 1.0 μg/kg (ppb). The aflatoxin concentrations of the remaining positive samples ranged from 1 to 10 μg/kg.

DISCUSSION

The total mold counts of the corn stored in the underground bin were undoubtedly affected by the removal of the caps from the sampling conduits. However, the percent of infected kernels was a better indication of the amount of mold activity in any given sample. Some samples with low mold counts had a high percentage of infected kernels. Thus, there was not good correlation between mold counts and the degree of mold invasion of kernels. The highest aflatoxin level (166 μg/kg) was found in a sample that had a high total mold count (10^6/g), high A. flavus count (10^5/g) and a high percentage of infected kernels (96%). The second highest level of aflatoxin (21 μg/kg) was found in a sample that had a moderately high total mold count (10^5/g) and percent infected kernels (75%) but a low A. flavus count (5/g). It is possible that the A. flavus level had declined by the time that the sample was taken. With other samples the levels of aflatoxins were low or not detected even though mold counts and/or percent infected kernels was high. Since a variety of molds were present in all of the samples, it is most likely that non-aflatoxigenic molds predominated and prevented the growth of A. flavus and aflatoxin.

In this study the corn stored in the underground storage facility tended to have higher total mold counts and higher percentages of mold infected kernels. However, the highest level of aflatoxin was found in corn from the above ground storage bin. The possibility that moisture may have gained access to the underground bin may have resulted in the greater growth of non-aflatoxigenic molds which competitively overgrew the A. flavus. Because of the possibility that the underground storage bin was vandalized and moisture apparently entered the bin it is not possible to make a valid comparison between the two storage methods.

SUMMARY

Fifty samples of corn (maize) stored in above and below ground storage facilities were examined for microbial content. Four culture media, employing two levels of A_w were used to detect fungal invasion. The media included potato dextrose agar plus tetracycline (PDAT), dichloran rose bengal chloramphenicol agar (DRBC), PDAT+7.5% NaCl and DRBC+7.5% NaCl. Surface sanitized corn kernels were placed directly on the surfaces of the agar media and the percent of infected kernels was determined. Mold and yeast counts were done using DRBC agar. Differential A. flavus-parasiticus counts were done using A. flavus-parasiticus agar (AFPA). Total mold counts ranged from $2x10^2$/g to $3x10^6$/g. Yeast counts were very low to none detected in most samples. Where yeasts had proliferated, mold counts were low. A. flavus-parasiticus counts were low with only a few samples having counts above 10^2/g. Internal mold contamination of the grain ranged from 0 to 97% of the kernels infected. DRBC and DRBC+salt were more effective in preventing the growth of spreader molds, and media with salt detected more infected kernels. Aflatoxin content of the stored corn ranged from none detected to 166 μg/kg. Most of the samples (54%) contained no detectable aflatoxin, one additional sample contained 21 μg/kg and the remaining positive samples contained from 1 to 10 μg/kg. Apparently, the molds in the majority of the samples were non-aflatoxigenic types and may have overgrown the aflatoxigenic A. flavus spp. While corn stored in the underground storage structure in general had higher mold counts and higher percent infected kernels, this was probably caused by moisture getting into the bin.

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Aflatoxin Occurrence in Virginia Price Support-Corn: 1986 Crop-Year

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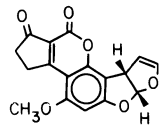
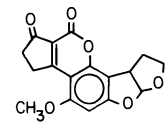
INTRODUCTION

Aflatoxins (AFTs), as representatives from a group of secondary fungal metabolites known as mycotoxins, are divided into several subgroups based on differing chemical characteristics (Figure 1). As a by-product of their metabolism, Aspergillus flavus and A. parasiticus produce AFTs including aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2). Collectively, the aflatoxins are among the most toxic hepatocarcinogens known (Sporn, 1966).

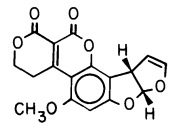
Aflatoxin-producing fungi are significant contaminants of some agricultural products. Corn, like many grain crops, is a target for the fungi and associated toxins (Golumbic and Kulik, 1969). In the United States, corn is an important feed grain for swine, cattle, and poultry (Jensen et al., 1977), and food for humans. The harmful effects of moldy feed have been documented in cattle (Schofield, 1924; Loosmore and Markson, 1961; Albright et al., 1964; Loosmore et al., 1964; Gopal et al., 1968; Lynch et al., 1970; Adamesteanu et al., 1974; Meronuck, 1981; Colvin et al., 1984). Similar responses in poultry (Blount, 1960; Chapman, 1961; Smith et al., 1976; Qin et al., 1983; Choudary, 1986) and in swine have been reported (Minne et al., 1964; Popescu et al., 1977; Kulcycki et al., 1982; Fiorentin et al., 1986). If aflatoxins occur in



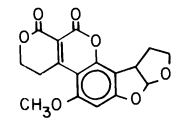
Aflatoxin B_1



Aflatoxin B_2



Aflatoxin G_1



Aflatoxin G_2

Figure 1. Structures of the Four Commonly Encountered Aflatoxins.

corn used as human food or in corn being fed to chickens, cattle, or swine to produce human food, there is a possibility of biocontamination, perhaps to levels high enough to cause human health concerns. It is generally accepted that if highly contaminated corn is used as animal feed, animal health will decline and AFT contamination could occur in the milk, meat, and eggs produced.

Mold growth is generally optimum at 30°C, 80-85% relative humidity (R.H.), and a substrate moisture content of 15-28%. Rough handling during harvest, the presence of moisture in storage areas, and heavy insect infestation can lead to increased fungal growth and higher AFT levels in stored corn (Spensley, 1963). Fungal presence does not necessarily mean that AFTs will be produced. Corn, peanuts, and soybeans all support fungal growth, but raw soybeans appear to be "resistant" to toxin production (Palmgren and Ciegler, 1983).

Assessment of AFT contamination has been accomplished by several quantitative analytical techniques. Ultraviolet spectrometry, thin-layer chromatography (TLC), and high-pressure liquid chromatography (HPLC) are acceptable techniques for the determination of AFT contamination. The current Association of Official Analytical Chemists (AOAC) methods utilize HPLC for mycotoxin quantification (Williams, 1984).

Virginia's climate has the potential to support both A. flavus and A. parasiticus, along with a virtually unending list of other fungi. In 1977, 28% of Virginia corn samples submitted to the State Division of Consolidated Laboratory Services were found to be contaminated with AFTs in concentrations exceeding the acceptable U.S. Food and Drug Administration (USFDA) tolerance level of 20 parts per billion (ppb) (Llewellyn and Katzen, 1981).

The Commonwealth of Virginia is ranked 21st of the 41 states producing corn for grain. This is 0.61% of U.S.A. corn production. However, in the Commonwealth, corn is third only to tobacco and soybeans as a cash crop (Dunkerly and Rowley, 1986).

The current report utilized data from the 1986 crop-year to examine AFT contamination in corn from the six climatological districts of Virginia. The AFT testing was required by the Agricultural Stabilization and Conservation Service of the United States Department of Agriculture (ASCS/USDA) as part of their price support program. Under the ASCS/USDA price support program, farmers were allowed to draw nine-month federal loans for the assessed value of their stored corn. Farmers had two options for loan repayment; repay the loan from the sale of the corn, or

allow the government to retain the corn in payment of the loan. This provided the farmers with money "up front" for their grain corn and allowed them to sell it elsewhere at a higher price during the nine-month loan period. In 1986, all farmers in Virginia who applied to ASCS/USDA for the price-support program loan were required to submit samples of their stored corn to their local ASCS/USDA office for AFT testing. The evaluation of AFT contamination and comparison with geography and precipitation deviations for the 1986 crop-year are reported in this study.

MATERIALS AND METHODS

The Mycotoxin Laboratory of the Virginia Division of Consolidated Laboratory Services began testing for AFTs in ASCS/USDA corn on September 1, 1978, at the request of the Marketing Division of the Virginia Department of Agriculture. In 1986, corn samples were submitted by the farmers to their county ASCS/USDA office. Aflatoxin analysis was conducted in a total of 60 counties/cities in the Commonwealth. These counties represent Virginia's corn belt and are listed in Table 1. Laboratory testing consisted of the solubilization and extraction of AFTs with acetone:water, 85:15(v:v). After further purification, the samples were stored over anhydrous Na_2SO_4 at $0^{o}C$ (Williams, 1984). The quantitation of AFB_1, AFB_2, AFG_1, and AFG_2 from the corn samples was determined by HPLC (Altex Chromatogram, 1978). The laboratory findings were given from 1 to "n" ppb AFT by subtype (AFB_1, AFB_2, AFG_1, and AFG_2). This procedure represented a more precise quantitative technique than TLC methods reported earlier (Llewellyn and Katzen, 1981).

The AFT data from the 1986 corn crop were grouped in the following categories in regards to total aflatoxin in the sample: 1) none detected; 2) less than 20 ppb; 3) 21 to 100 ppb; 4) 101 to 400 ppb; 5) greater than 400 ppb. Corn in groups 1 and 2 is allowed for human consumption. Corn in group 3 may be fed to mature cattle, swine, and poultry. Corn in groups 4 and 5 should not be fed to any animal. Corn assayed in this study came exclusively from Virginia. Not all of the 136 counties/cities of Virginia are represented due to low corn production in certain geographical locales.

Monthly precipitation totals for the six climatological regions in Virginia for 1986 were obtained from the 1986 National Oceanic and

Table 1. Virginia Counties/Cities Submitting Corn Samples for Aflatoxin Analysis in 1986.

Counties:District	Total Samples Submitted	Samples Positive[a]
Accomack/Northampton Counties:TW/TW	15	14
Albemarle County:WP	1	0
Amelia County:EP	1	0
Augusta County:CM	10	5
Campbell County:WP	1	1
Caroline County:N	32	21
Charlotte County:WP	2	0
Chesapeake City:WP	55	54
Clarke County:N	2	1
Culpeper County:N	21	5
Dinwiddie County:CP	5	5
Essex County:TW	24	24
Fauquier/Prince William Counties:N/N	7	1
Fluvanna County:CP	2	1
Frederick County:N	5	1
Gloucester County:TW	1	0
Goochland County:CP	7	2
Greensville County:TW	13	11
Halifax County:WP	1	1
Hanover County:CP	21	14
Isle of Wight County:TW	49	46
James City County:TW	1	1
King George/Stafford Counties:TW/N	16	9
King and Queen/King William Counties:TW/TW	16	12
Lancaster County:TW	8	8
Loudoun/Fairfax Counties:N/N	24	0
Louisa County:CP	14	0
Lunenburg County:CP	3	3
Madison County:N	24	4
Middlesex County:TW	11	10
Nelson County:WP	1	0
New Kent/Charles City/Henrico Counties: TW/TW/CP	34	33
Nottoway County:CP	1	1
Northumberland County:TW	13	13
Orange County:N	6	1
Page County:N	14	1
Patrick County:CM	3	0
Powhatan County:CP	5	0
Prince George County:TW	23	22

(cont.)

Table 1. Continued.

Rappahannock County:N	1	0
Rockbridge County:CM	1	0
Richmond County:TW	17	17
Rockingham County:CM	10	1
Scott County:SM	1	0
Shenandoah County:N	3	0
Southampton County:TW	128	117
Spotsylvania County:N	2	0
Suffolk City:TW	138	132
Surry County:TW	3	3
Sussex County:TW	18	16
Warren County:N	3	1
Washington County:SW	1	0
Westmoreland County:TW	42	35
TOTAL: 60	860	647

TW[b]= 22; N= 16; EP= 10; WP= 6; CM= 4; SM= 2

[a]No. of samples testing positive for total AFT. i.e., greater than 1 ppb -the limit of detection.

[b]TW,N,EP,WP,CM, and SM are climatological districts as explained in the text.

Atmospheric Administration's (NOAA) Climatological Data monthly publication. The two data groups, aflatoxin occurrence and rainfall, were studied for trends which could offer an explanation for the regional differences in AFT contamination for Virginia corn in 1986 .

RESULTS AND DISCUSSION

Climatological Data

The Commonwealth of Virginia was divided into six geographical districts for precipitation analysis by NOAA. The districts were Tidewater (TW), Eastern Piedmont (EP), Western Piedmont (WP), Northern (N), Central Mountain (CM), and Southwestern Mountain (SM) (Figure 2). The 1986 precipitation deviations are presented in Table 2. The data represented in Table 2 are monthly, cumulative annual, and cumulative crop-year rainfall deviations for each climatological district. The precipitation deviations are given in inches per unit time. From Table 2, it can be seen that the TW district had the greatest annual and crop-year rainfall deficits of the six districts at -12.2 and -9.7 inches, respectively. The remaining districts had rainfall deficits significantly less than the TW district.

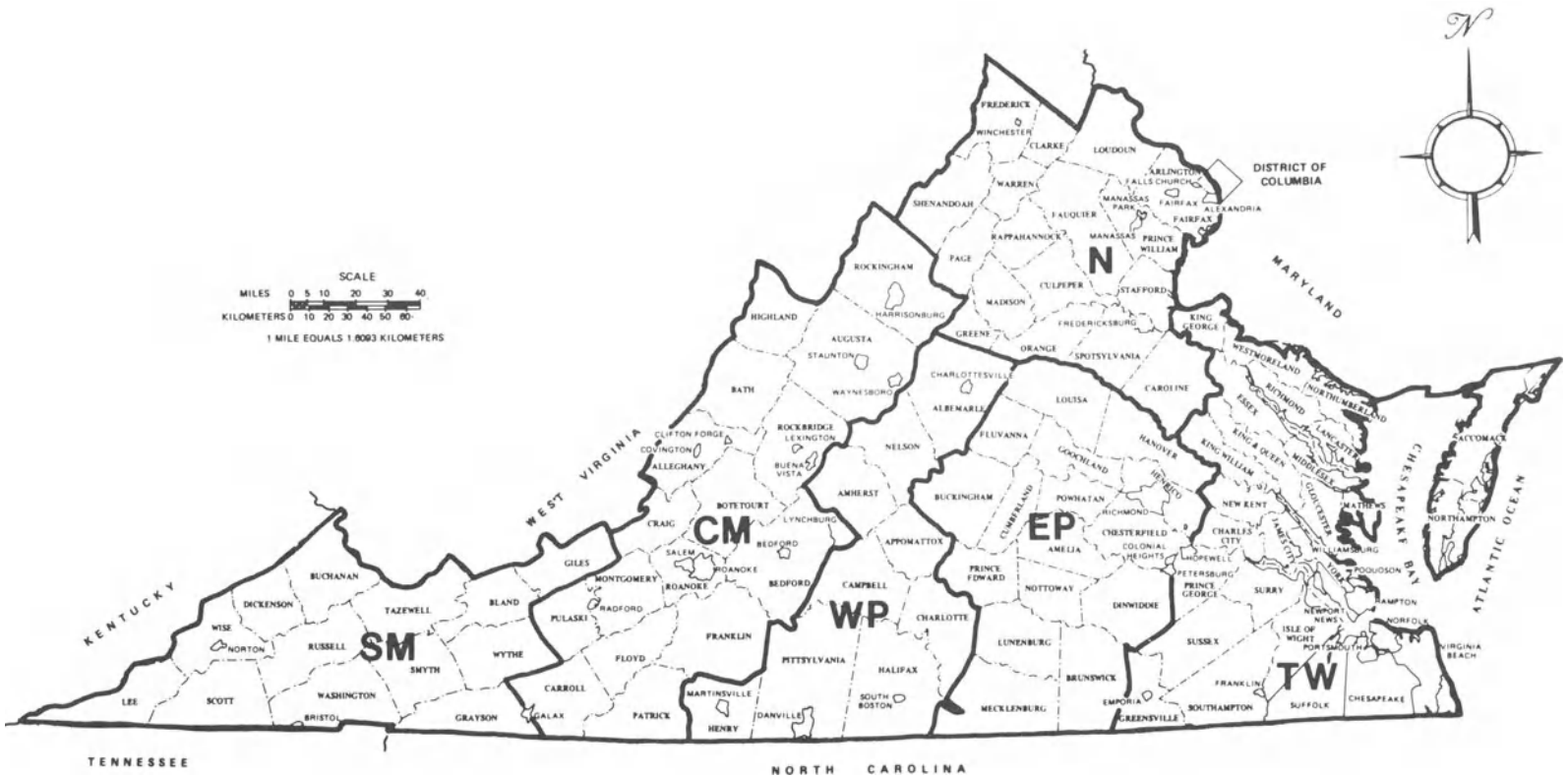


Figure 2. The Climatological Districts of Virginia are: Tidewater, (TW); Eastern Piedmont, (EP); Western Piedmont, (WP); Northern, (N); Central Mountain, (CM); Southwestern Mountain, (SM).

Table 2. Precipitation Deviations from Normal (30-year mean)[a] for the Six Climatological Districts in Virginia for 1986[a].

	Climatological District					
Month[b]	TW[c]	EP[d]	WP[e]	N[f]	CM[g]	SM[h]
January	-0.765	-1.084	-2.129	-1.262	-1.755	-2.039
February	-0.814	-0.637	-0.427	+0.674	-0.375	+0.236
March[b]	-2.672	-1.508	-2.544	-1.768	-1.568	-1.975
April[b]	-1.293	-1.686	-2.077	-0.553	-0.983	-2.164
May[b]	-1.767	-0.977	-1.171	-2.082	-0.643	+1.382
June[b]	-2.068	-2.817	-2.543	-2.294	-1.426	-2.150
July[b]	-0.263	-0.089	-0.869	-0.095	-0.867	-0.806
August[b]	+1.277	+1.962	+1.558	+0.463	-0.670	+1.009
September[b]	-3.360	-2.630	-1.263	-2.234	-1.096	-0.190
October[b]	-1.497	-0.797	-0.885	-1.917	-1.791	-0.653
November[b]	-0.743	-0.067	+0.962	+2.045	+1.414	+1.684
December	+1.768	+1.248	+1.903	+1.918	+1.269	+1.307
Cumulative Deficit for Year (12 months)	-12.197	-9.082	-9.485	-7.105	-8.491	-4.359
Cumulative Deficit for Crop Year (8 months)	-9.714	-7.101	-6.288	-6.667	-6.062	-1.888

[a]Precipitation Totals were obtained for each district from the National Oceanic and Atmospheric Administration, Climatological Data 1986 Monthly publication. The monthly precipitation deviations are mean monthly precipitation totals for all collecting sites in the district - the mean normal precipitation for all collecting sites in the district. - values represent a rainfall deficit, + values represent rainfall excess, as compared to a 30 year mean. All units are in inches/time interval given.

[b]Crop-Year Month

[c]Tidewater Climatological District

[d]Eastern Piedmont Climatological District

[e]Western Piedmont Climatological District

[f]Northern Climatological District

[g]Central Mountain Climatological District

[h]Southwestern Mountain Climatological District

Corn Production/AFT Contamination Data

According to the Virginia Agricultural Statistics Bulletin 1986, as compiled by the Virginia Agricultural Statistics Service, the two data sets presented for corn were corn for grain and corn for silage. Grain corn is typically a higher-quality product than silage corn. In 1986, there were 730,000 acres of corn planted in the Commonwealth, of which, 400,000 were harvested as grain corn, and 300,000 were harvested as silage. Grain corn yields averaged 54.0 bushels/acre and silage corn averaged 10 tons/acre. Production values were $36,720,000 and $80,760,000, respectively. The corn tested in this study was grain corn. Grain corn can be used for corn meal, cereals, and corn chips, as well as animal feed.

Each county submitting corn samples in 1986 is presented in Table 1. The total number of samples analyzed is shown. A state-wide and district analysis of AFT contamination is provided in Table 3. Counties in each district submitted samples for toxin analysis. All counties in the TW region submitted some samples contaminated with AFTs greater than 20 ppb, while low levels of AFT contamination were seen in the EP and N districts. The WP, CM, and SM districts showed no samples contaminated with AFTs greater than 20 ppb. The counties submitting samples with greater than 20 ppb AFT are ranked in Table 4. The district is provided with the percent of the samples contaminated greater than 20 ppb. The counties submitting 10 or more samples are ranked in Table 5.

Of the 860 samples received and tested, 647 were found to be positive for one of the four AFTs (> 1 ppb -limit of detection). Of these positive samples, 384 or 59% came from five localities: the counties of Southampton, Isle of Wight, and Westmoreland, and the cities of Suffolk and Chesapeake. Ninety-three percent of the samples submitted from these five localities were found to be positive for AFT contamination. Samples from these localities accounted for 48% of the total number of samples submitted throughout the state. Four of the five localities are located in the southeast corner of the state. Westmoreland County is located on the Northern Neck of Virginia. All five are in the TW district. Other Northern Neck localities had fewer total samples, but high percentages of detectable AFT contamination (Northumberland County, 13 of 13; Richmond County, 17 of 17; Essex County, 24 of 24; Accomack/Northampton (collectively) on the Eastern Shore, 14 of 15; Lancaster County, 8 of 8; and Middlesex County, 10 of 11. These are the number of samples with AFT greater than 1 ppb). The three highest levels of total AFT contamination

Table 3. Summary of Virginia Aflatoxin Contamination in Price-Support Corn for the 1986 Crop-Year.

State-wide Aflatoxin Contamination

Total Number of Counties/Cities (95/41) = 136

Total Number of Counties/Cities submitting samples for aflatoxin testing = 60/136 or 44%

Total number of Counties/Cities with aflatoxin contamination greater than 20 ppb = 27/136 or 20%

Virginia Climatological District Contamination

Tidewater District (TW)
Number of Counties Reporting = 20

Number of Counties Reporting
AFT levels greater than 20 ppb = 20 or 100%

Northern District (N)
Number of Counties Reporting = 14

Number of Counties Reporting
AFT levels greater than 20 ppb = 5 or 35.7%

Eastern Piedmont District (EP)
Number of Counties Reporting = 10

Number of Counties Reporting
AFT levels greater than 20 ppb = 2 or 20%

Western Piedmont District (WP)
Number of Counties Reporting = 6

Number of Counties Reporting
AFT levels greater than 20 ppb = 0 or 0%

Central Mountain District (CM)
Number of Counties Reporting = 4

Number of Counties Reporting
AFT levels greater than 20 ppb = 0 or 0%

Southwest Mountain District (SM)
Number of Counties Reporting = 2

Number of Counties Reporting
AFT levels greater than 20 ppb = 0 or 0%

Table 4. Ranking of All Virginia Counties/Cities Submitting Samples Having Greater Than 20 ppb Aflatoxin During 1986.

County/City	District	Number of Samples (n)	Contaminated Samples (%)	Ranking
James City	TW[a]	1	100	1
Northumberland	TW	13	85	2
Lancaster	TW	8	75	3
Accomack	TW	15	73	4
Middlesex	TW	24	73	5
Isle of Wight	TW	49	69	6
Surry	TW	3	66	7
Emporia	TW	13	62	8
Southampton	TW	128	62	9
New Kent[b]	TW	34	59	10
Essex	TW	24	54	11
Westmoreland	TW	42	52	12
Clarke	N[c]	2	50	13
Suffolk	TW	138	50	14
Chesapeake	TW	55	47	15
Richmond	TW	17	47	16
King and Queen[d]	TW	16	44	17
Prince George	TW	23	39	18
Sussex	TW	18	28	19
Dinwiddie	EP[e]	5	20	20
Fredrick	N	5	20	21
King George	TW	5	20	22
Caroline[f]	N	32	16	23
Fauquier[f]	N	7	14	24
Hanover	EP	21	14	25
Culpeper	N	21	10	26

[a]TW, Tidewater Climatological District

[b]Counties submitting samples to the New Kent ASCS office were New Kent, Charles City, and Henrico. New Kent samples are a combination of the three counties.

[c]N, Northern Climatological District

[d]Counties submitting samples to the King and Queen ASCS office were King and Queen and King William. King and Queen samples are a combination of the two counties.

[e]EP, Eastern Piedmont Climatological District

[f]Counties submitting samples to the Fauquier ASCS office were Fauquier and Prince William. Fauquier samples were considered a combination of both counties.

Table 5. Ranking of All Virginia Counties/Cities Submitting Samples Having Greater Than 20 ppb Aflatoxin During 1986 and More Than 10 Samples Tested.

County/City	District	Number of Samples (n)	Contaminated Samples (%)	Ranking
Northumberland	TW[a]	13	85	1
Accomack	TW	15	73	2
Middlesex	TW	24	73	3
Isle of Wight	TW	49	69	4
Emporia	TW	13	62	5
Southampton	TW	128	62	6
New Kent[b]	TW	34	59	7
Essex	TW	24	54	8
Westmoreland	TW	42	52	9
Suffolk	TW	138	50	10
Chesapeake	TW	55	47	11
Richmond	TW	17	47	12
King and Queen[c]	TW	16	44	13
Prince George	TW	23	39	14
Sussex	TW	18	28	15
Caroline	N[d]	32	16	16
Hanover	EP[e]	21	14	17
Culpeper	N	21	10	18

[a]TW, Tidewater Climatological District

[b]Counties submitting samples to the New Kent ASCS office were New Kent, Charles City, and Henrico. New Kent samples are a combination of the three counties.

[c]Counties submitting samples to the King and Queen ASCS office were King and Queen and King William. King and Queen samples are a combination of both counties.

[d]N, Northern Climatological District

[e]EP, Eastern Piedmont Climatological District

were found in samples from Suffolk (2100 ppb AFT, 1900 ppb AFT, and 1800 ppb AFT). The thirty highest AFT contaminated samples and the counties and districts from which they were submitted are given in Table 6. Of the 30 samples with the highest AFT contamination levels, all were from the 12 areas listed above, with the exception of three samples which were from King and Queen and King William counties. The range of the 30 highest AFT contaminated samples was from 250 to 2,100 ppb and all 30 samples were submitted from counties/cities in the TW district. The subtypes of AFT detected were predominantly AFB_1 with less AFB_2, and only slight occurrences of AFG_1 and AFG_2. Of the 860 samples tested, 647 were positive for AFTs. Of these 647 samples, 111 were shown to contain levels of AFT above 100 ppb. Samples in which AFTs were detected at greater than 20 ppb totaled 365, or 56%.

The counties with mean AFT levels in submitted corn samples equal to or greater than 20 ppb are given in Figure 3. Counties with mean AFT contamination levels equal to or greater than 80 ppb are given in Figure 4. With the exception of Henrico and Dinwiddie counties (CP district), all the counties with AFT contamination levels equal to or greater than 20 ppb were from the TW district. All counties with mean AFT levels equal to or greater than 80 ppb were from the TW district.

For the 1986 corn crop in general, and considering all the data on a state-wide basis, the following percentages of contaminated samples fell into the following level of contamination categories: For category 1 (none detected), 25% of the samples tested occurred in this group; category 2 (< 20 ppb), 33.5% of the samples tested occurred in this group; category 3 (21 to 100 ppb), 29.6% of the samples tested occurred in this group; category 4 (101 to 400 ppb), 10.0% of the samples occurred in this group; and category 5 (> 400 ppb), 1.9% of the samples tested occurred in this group. From this data, it is evident that 58.5% of the corn tested could not be consumed by humans, and 11.9% of the corn could not be used as animal feed. Under the AFT contamination guidelines mentioned previously, 11.9% of the crop was lost as based on these samples. These levels were significantly higher than the values 18.9% and 3.2% respectively, reported by Llewellyn and Katzen for the 1977 corn crop.

From the available data, represented in Tables 2 and 3, it appears that the crop-year rainfall deficit is directly related to increased AFT levels (Table 7). It seems that the greater the rainfall deficit the greater the extent of AFT contamination.

Table 6. The Thirty Highest Aflatoxin Contaminated Samples with AFT by Subtype and Total Aflatoxin Content.

County/City	Aflatoxin Quantities in ppb					Climatological District
	AFB_1	AFB_2	AFG_1	AFG_2	Total	
Suffolk	2000	110	16	1.1	2100	TW[a]
Suffolk	1700	140	22	5.5	1900	TW
Suffolk	1600	120	35	6.7	1800	TW
King and Queen	770	64	9.7	2.0	850	TW
Isle of Wight	700	21	57	5.4	780	TW
Southampton	670	39	33	3.9	750	TW
Southampton	670	24	2.8	0.4	700	TW
King and Queen	590	50	4.3	2.8	650	TW
Richmond	420	150	32	4.6	610	TW
Chesapeake	480	28	ND	ND	510	TW
Isle of Wight	480	14	0.7	ND	490	TW
Southampton	440	21	14	1.2	480	TW
Westmoreland	450	17	11	0.6	480	TW
Accomack/Northampton	420	2.1	4.6	1.3	450	TW
Westmoreland	400	19	11	1.4	430	TW
Westmoreland	380	16	18	5.5	420	TW
Accomack/Northampton	370	20	7.2	1.1	400	TW
Isle of Wight	370	16	7.2	1.3	390	TW
King and Queen	330	37	7.5	1.8	380	TW
Accomack/Northampton	350	20	0.5	ND	370	TW
Isle of Wight	340	11	4.5	ND	360	TW
Isle of Wight	340	9.8	4.6	ND	350	TW
Lancaster	310	17	ND	ND	330	TW
Southampton	300	17	5.1	3.0	330	TW
Accomack/Northampton	290	17	6.8	1.2	310	TW
Accomack/Northampton	280	9.8	15	1.6	310	TW
Suffolk	290	12	1.1	0.5	300	TW
Suffolk	220	7	44	3.3	270	TW
Chesapeake	250	10	1.8	0.2	260	TW
Southampton	210	11	25	2.0	250	TW

[a]Tidewater Climatological District

(ND) None Detected (AFT less than 1 ppb)

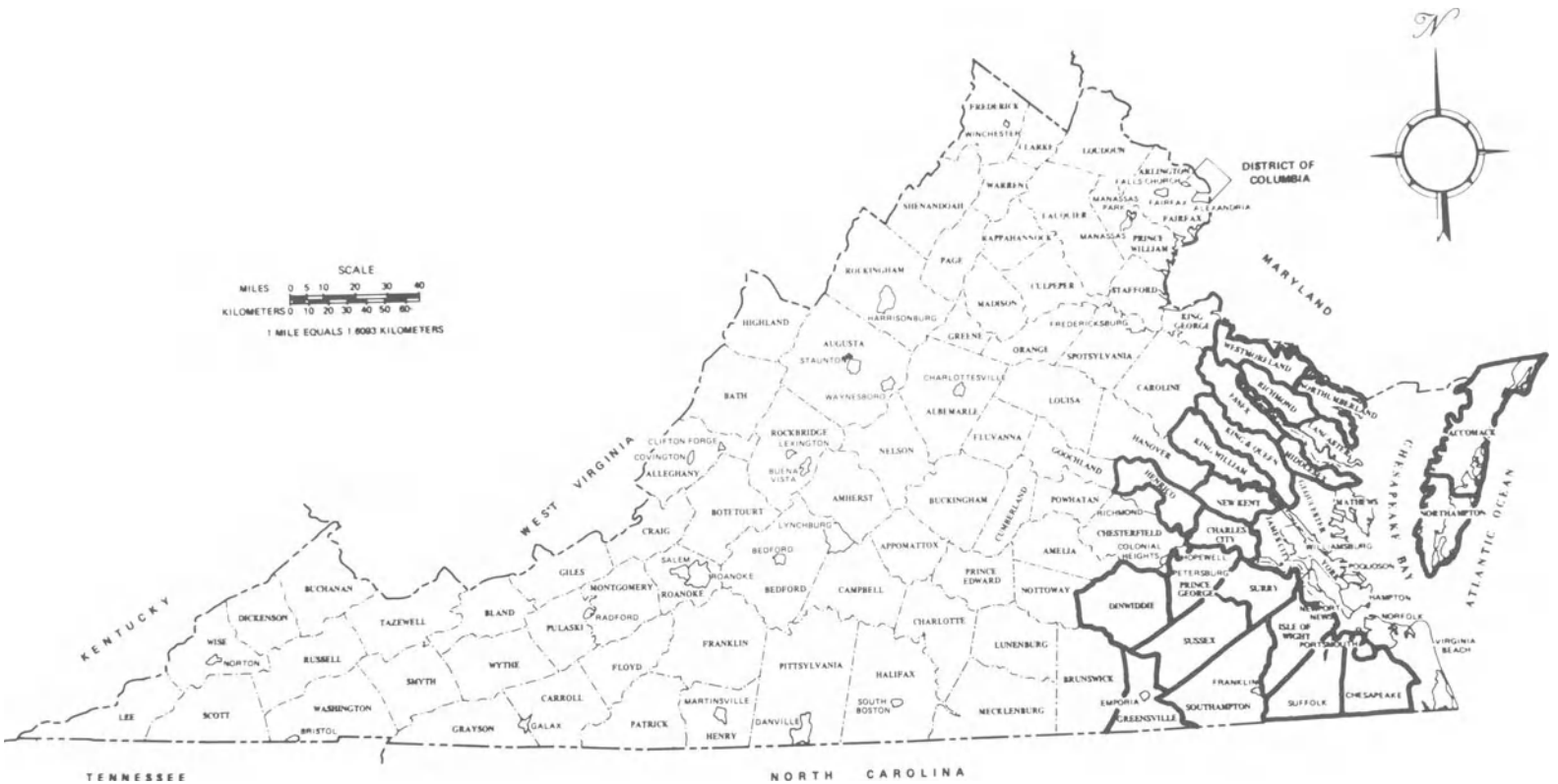


Figure 3. Counties/Cities Having Mean Total Aflatoxin Levels Equal to or Greater Than 20 ppb for the 1986 Crop-Year.

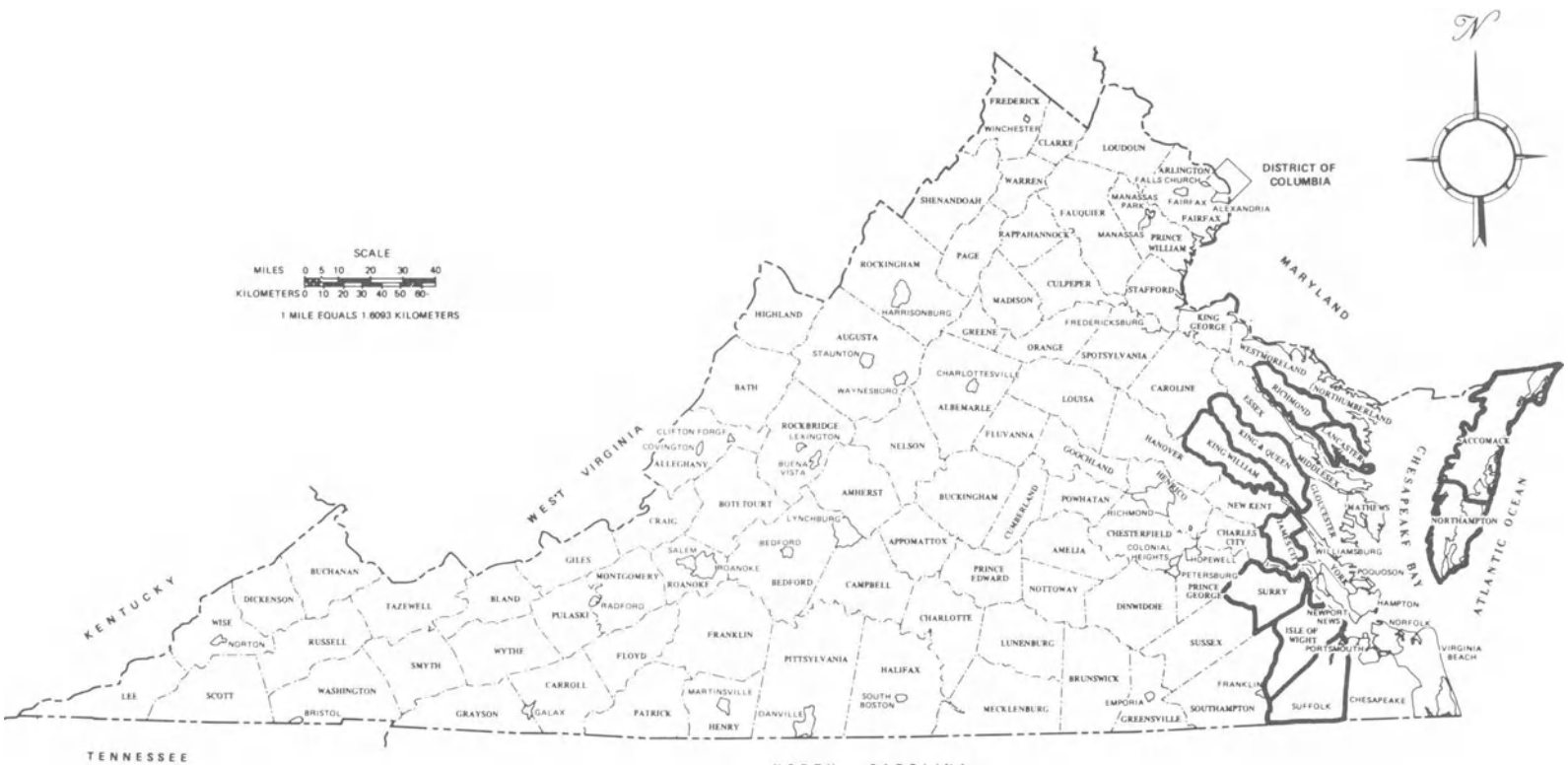


Figure 4. Counties/ Cities Having Mean Total Aflatoxins Equal to or Greater Than 80 ppb for the 1986 Crop-Year.

Table 7. Climatological District Crop-Year Rainfall Deficits and AFT Contamination in 1986 ASCS/USDA Price Support Corn for 1986.

Climatological District	Rainfall Deficit[a]	% of Region Contaminated[b]
Tidewater	-9.714	100.0
Eastern Piedmont	-7.101	20.0
Northern	-6.667	35.7
Western Piedmont	-6.228	0.0
Central Mountain	-6.062	0.0
Southwestern Mountain	-1.888	0.0

[a]Crop-Year (April-November) Rainfall Deficit in inches.

[b]Percent of counties in the climatological district submitting samples contaminated with AFT equal to or greater than 20 ppb.

The geographical district involved also may be a contributing factor for substantial exposure to higher levels of humidity after the crop is harvested and this, in part, may be responsible for the district differences in AFT contamination. AFT contamination was the highest in the TW, N, and EP districts. These districts generally exhibit higher R.H. than do the WP and CM districts. In general, the SM district had a high R.H., but contributed so few samples to the ASCS/USDA that little can be determined concerning the district's ability to support fungal growth and subsequent AFT production.

In the Commonwealth, 73% of the counties which submitted samples contaminated with AFT greater than or equal to 20 ppb were in the TW climatological district. Also, all 30 samples with the highest levels of AFT were all submitted from the TW district. Considering this, it would seem that the Tidewater district, with its rivers, marshlands, and tributaries would provide a contributory environ. Also, with it's tendancy for higher humidity and year-round warmth during crop storage, the TW district could be more supportive of AFT-producing fungi than other climatological districts in the state.

Previous studies propose high rainfall levels to be an important factor in mold growth and toxin production. In this study, geographic data suggesting that high levels of ambient R.H. during storage may also be a predictor factor for AFT contamination that cannot be ruled out. It is unfortunate that accurate daily humidity and soil moisture for each climatological district in the state are not readily available. Based on the state-wide ASCS/USDA corn AFT analysis and the rainfall deficit

calculations for the six climatological districts of the state, there is a definite relationship between the increased stress from "drought" as measured by rainfall deficit, and the percent of corn samples found to be contaminated with AFT. Rainfall deficits at these levels in the future would be expected to forecast similar occurrence of AFTs including possible similar quantitative levels for the AFT positive districts in the state. The use of this data as a predicting procedure does have many shortcomings, but the positive relationship between "drought" and AFT occurrence and levels are nevertheless apparent. Possibly, irrigation could be a procedure worth considering if rainfall deficits are noted. Such a drought stress response in combination with natural high ambient humidity is further supported by the proposal of Dorner et al. (1989), who theorize that kernel water activity in peanuts is associated with the capacity of the kernels to produce phytoalexins. Phytoalexins seem to confer some resistance to contamination by AFTs. When drought conditions occur the kernel production of phytoalexins was shown to decrease. Soon after the decrease in phytoalexins there was an appearance of AFT contamination. Thus providing a link in peanuts between drought stress and AFT contamination.

SUMMARY

Stored corn from the 1986 corn harvest in Virginia showed a geographical distribution of AFT contamination, with the highest levels and extent of contamination in the Tidewater climatological district. High rainfall deficits and high R.H. for that area during crop storage appears to play a role in the occurrence of AFTs. This occurrence correlates well with the geographical distribution of AFT contamination. Areas with smaller rainfall deficits and lower R.H. in Virginia exhibited substantially less AFT contamination. It would seem that the contributory factors for AFT contamination in Virginia might be: 1) drought-like conditions as measured by rainfall deficits 2) high R.H. 3) poorly ventilated and controlled storage areas 4) and temperature. Specifically for this crop year, 58.5% of the corn exceeded the level for use for human consumption, and 11.9% of the corn exceeded the acceptable level for use as animal feed. These levels were significantly higher than the values of 18.9% and 3.2% reported by Llewellyn and Katzen for the 1977 corn crop.

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Aflatoxin M_1 Contamination of Milk Samples in Virginia During 1981 to 1987

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INTRODUCTION

Aflatoxin B_1 (AFB_1), when fed to dairy cattle, has been shown to yield the milkborne metabolite aflatoxin M_1 (AFM_1)(Figure 1). AFM_1 carry over into milk via the secretory route is low (2% $\pm$ 1% of AFB_1 introduced), but has been shown to be increased by treatment with polyhalogenated biphenyls (Heeschen and Bluethgen, 1989). Higher levels of AFB_1 grain contamination in feed thus yields higher levels of AFM_1 in the milk produced (Zimmerli and Blaser, 1979). AFM_1 has been shown to be both toxic and carcinogenic (Hsieh et al., 1986). Even though AFM_1 is less toxic when compared to its unoxidized precursor, AFB_1, the risks that are posed to humans through the consumption of dairy products warrant concern. The toxicity and related carcinogenicity of AFM_1 has prompted the testing of milk and associated products for the its presence and is addressed via the U.S. FDA (Federal Regulation) action level of 0.5 ppb AFM_1. Methods for quantifying aflatoxin include, thin-layer chromatography (TLC), high pressure liquid chromatography (HPLC), and monoclonal antibody technology. The current Association of Official Analytical Chemists methods for analysis utilizes HPLC to quantify aflatoxins (Williams, 1984).

Milk samples from Virginia were tested by the State Division of Consolidated Laboratory Services. The results from these milk analyses for the years 1981-1987 are presented with commentary and serve as a chronological record for future epidemiological studies.

MATERIALS AND METHODS

In Virginia, milk testing for AFM_1 has been conducted by the Virginia Division of Consolidated Laboratory Services. The milk samples were gathered by state milk inspectors. Samples came directly from dairies, from milk carriers, and from the local marketplace. Both raw and pasteurized/homogenized samples were tested. The isolation, purification, and quantitative analysis of AFM_1 from milk was conducted in accordance with the Official Methods of Analysis of the Association of Official Analytical Chemists (Williams, 1984).

RESULTS AND DISCUSSION

Table 1 provides an annual synopsis of AFM_1 contamination for the calendar years 1981 through 1987. The year, number of samples analyzed (N), the number and percent of samples with no AFM_1 detected, the number and percent of samples with <0.5 ppb AFM_1, and the number and percent of samples with $\geq$0.5 ppb are given. Total values for the seven years examined are also included. The data describing AFM_1 levels is presented by two numbers in the tables. The first value reports the number of samples in the respective grouping, the second value reports the relative percentage for the preceding number when compared to the total number of samples analyzed.

Table 2 provides monthly AFM_1 contamination values for the seven years examined. This table has a similar format as used in table 1 with the exception of the years, months replace years in table 2.

In Table 1, 1984 appears to be the most severe year for milk contamination. During that year, 9, or 1.8%, of the samples tested had AFM_1 levels equal to or greater than 0.5 ppb. The remaining years studied had no samples exceeding 0.5 ppb. The category for contamination levels less than 0.5 ppb was ranked and the three highest contamination years were, 1984, 142 or 28.6%; 1987, 17 or 15.3%; and 1983, 35 or 13.5%.

From Table 2, which summarizes monthly contamination over the seven year study, January and February show contamination greater than 0.5 ppb as do both May and June. This may be due to the high degree of maintenance of animals on stored feed. The number of samples with lower level contamination, less than 0.5 ppb, peaked in April and in October. The results suggest a biannual cyclic pattern of AFM_1 contamination in milk. It is interesting to note that this same annual bimodality appears in corn analyzed for AFB_1 (Mooney et al., 1990). As expected, the information presented suggests that years in which grain stores were

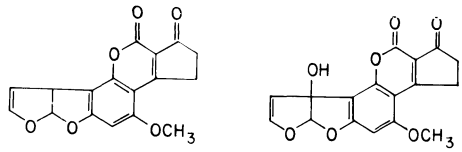

Figure 1. Structure of Aflatoxin B_1 and Its Metabolite, Aflatoxin M_1.

Table 1. Aflatoxin M_1 Levels in Milk Samples by Year (1981-1987).

Year	N	None Detected and Percentage	<0.5 ppb and Percentage	≥0.5 ppb and Percentage
1981	346	326/94.2%	20/ 5.8%	0/ 0.0%
1982	320	308/96.3%	12/ 3.8%	0/ 0.0%
1983	259	224/86.5%	35/13.5%	0/ 0.0%
1984	497	346/69.6%	142/28.6%	9/ 1.8%
1985	377	338/89.7%	39/10.3%	0/ 0.0%
1986	376	346/92.0%	30/ 8.0%	0/ 0.0%
1987	111	94/84.7%	17/15.3%	0/ 0.0%
Total	2286	1982/86.7%	295/12.9%	9/ 0.4%

Table 2. Aflatoxin M_1 Levels in Milk Samples by Month (1981-1987).

Month	N	None Detected And Percentage	<0.5 ppb And Percentage	≥0.5 ppb And Percentage
Jan	252	230/91.3%	20/ 7.9%	2/ 0.8%
Feb	308	247/80.2%	56/18.2%	5/ 1.6%
Mar	258	225/87.2%	33/12.8%	0/ 0.0%
Apr	136	104/76.5%	32/23.5%	0/ 0.0%
May	268	240/89.6%	27/10.1%	1/ 0.4%
Jun	147	134/91.2%	12/ 8.2%	1/ 0.7%
Jul	130	117/90.0%	13/10.0%	0/ 0.0%
Aug	86	82/95.4%	4/ 4.6%	0/ 0.0%
Sep	203	197/97.0%	6/ 3.0%	0/ 0.0%
Oct	99	70/70.7%	29/29.3%	0/ 0.0%
Nov	198	161/81.3%	37/18.7%	0/ 0.0%
Dec	201	175/87.1%	26/12.9%	0/ 0.0%
Total	2286	1982/86.7%	295/12.9%	9/ 0.4%

more highly contaminated with AFTs yield higher levels of the AFB_1 metabolite in milk. Children are often considered the primary consumer at risk (Bennett et al., 1980). Exposure to AFM_1 would seem to be more widespread than exposure to AFB_1 because of the ubiquitous nature of milk and milk products in normal diets (Stoloff, L., 1980). Although AFM_1 is considered less carcinogenic than its precursor (AFB_1) (Vesely and Vesala, 1983) the public health concern and especially the citizen response to carcinogens in food needed by children encourages broad spectrum testing of milk products for its presence and strict adherence to the U.S. FDA maximum tolerance level.

SUMMARY

In general, AFM_1 contamination of milk and milk products in Virginia as monitored by the Virginia Division of Consolidated Laboratory Services does not appear to be a significant problem. Of the seven years examined, only 1984 had samples contaminated at levels equal to or greater than 0.5 ppb AFM_1. This represented 1.8% of the samples. Analysis of monthly contamination gave a bimodal distribution of highly contaminated samples. this distribution correlated well with similar distribution of contamination levels in grain. For the six years studied 0.4% out of 2,286 samples were equal to or above 0.5 ppb. Although the ideal level would be no reports for this level, 0.4% supports, in our opinions, notable control of milk/milk products reaching the public and is likely to be a negligible public health concern.

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Aflatoxin Occurrence in Samples of Commercial Corn Meal Sold in Virginia: 1983-1986

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INTRODUCTION

Corn products which reach consumers on a daily basis have been shown to contain mycotoxins including the highly carcinogenic aflatoxins (AFTs) (Nout and Saint Hilaire, 1983). The U.S. Food and Drug Administration (USFDA) has set 20 ppb as an action level. AFTs in corn products at levels greater than 20 ppb are not allowed for human consumption. These toxins survive most commercial food processing treatments, but ammonification treatments have shown success in reducing the levels of AFTs found in processed corn products (Brekke et al., 1978; Nofsinger and Anderson, 1979).

Aflatoxins are secondary metabolites isolated from several Aspergillus-fungi, most notably A. flavus and A. parasiticus (Sporn et al., 1966). Corn products are particularly susceptible to AFT contamination because they are generally derived from corn stored in grain elevators or silos. These grain elevators, if poorly maintained, could provide an excellent environ for the growth and sporulation of Aspergillus fungi. Subsequent production of AFTs by these fungi has been documented (Nout and Saint-Hilaire, 1983). Pre-harvest corn may also contribute to the toxin levels.

The concern to protect human health has prompted the testing and regulation of corn and related products for AFTs. In this study, commercial corn meal samples were routinely collected and analyzed to determine the quantities of AFTs present following production treatments.

MATERIALS AND METHODS

Commercial corn meal was purchased as part of a routine check program and analyzed by the Food Laboratory of the Virginia Division of Consolidated Laboratory Services. The quantities of AFTs in parts per billion (ppb) were determined by high pressure liquid chromatography (HPLC) in accordance with the Association of Official Analytical Chemists standards (AOAC) (Williams, 1984). Those results from 1983 through 1986 are reported herein. Table 1 describes the procedure followed for AFT analysis.

RESULTS AND DISCUSSION

The results of tests undertaken by the Food Laboratory for the years 1983 through 1986, suggest reason for concern regarding human exposure to AFTS. AFT exposures, exceeding the USFDA maximum acceptable level for human food (20 ppb AFT), due to the consumption of corn meal products may have occurred. On an average, over the four years evaluated in this study, 43.8% of the corn meal analyzed contained detectable levels of AFTs and 8.1% of the samples tested had AFTs at levels greater than 20 ppb.

The annual results of AFT analysis of these samples for the years 1983-1986 are as follows (Table 2): Of the 599 samples tested in 1983, 220 or 36.7% showed detectable levels of aflatoxin (AFTs). Of these 220 samples with detectable levels of AFTs, 30 or 5% were found to have AFT levels greater than 20 parts per billion (ppb). On a monthly basis, the highest extent of contamination was found in the December tests (n=240) with 126 or 52.5% of the samples showing detectable levels of aflatoxins. Of the 391 samples tested in 1984, 224 or 57.3% had detectable levels of AFTs. Of the 224 samples, 46 or 11.8% were found to have AFTs greater than 20 ppb. The highest percentage of contamination was found in the August tests (n=61) with 42 or 68.8% of the samples showing detectable levels of aflatoxins. Of the 268 samples tested in 1985, 98 or 36.6% had detectable levels of AFTs. Of the 98 samples, 35 or 13.1% were found to have AFTs greater than 20 ppb. The highest percentage of contamination was found in the May tests (n=28) with 23 or 82.2% of the samples showing detectable levels of AFTs.

Of the 388 samples tested in 1986, 180 or 46.4% had detectable levels of AFTs. Of the 180 samples, 23 or 5.9% were found to have AFTs greater than 20 ppb. The highest percentage of contamination was found in the November tests (n=63) with 36 or 57.2% of the samples showing detectable levels of AFTs. Of the four years examined in this study, 1984 appears to have had the highest level of contamination while 1985 had the highest percentage of corn meal samples unfit for human consumption under USFDA guidelines.

Table 1. Method of Analysis for Corn Meal Aflatoxins (AFTs).

Extraction[a]

AFTs were extracted from corn meal with acetone-water (85:15) and filtered. (solution A).

Purification[a]

To 150 ml of solution A was added 3g basic $CuCO_3$ (solution B). Mix 170 ml 0.2 N NaOH and 30 ml $CuCO_3$ slurry (made from adding 20g anhyd. $FeCl_3$ to 250ml water) (solution C). Mix solution B and solution C (solution D). To solution D, add 150 ml diat. earth, mix well, and filter with Whatman No. 4 (solution E).

Transfer 150ml solution E to a 500ml separator; add 150ml 0.03% H_2SO_4 and 10ml $CHCl_3$; shake for 2 min. Collect CHCl3 phase and store over anhyd. Na_2CO_3 (solution F).

Chromatography[b]

High pressure liquid chromatography was used to determine aflatoxin levels by subtype for solution F.

[a]Adopted from Williams, S. Official Methods of Analysis, Assoc. Offic. Anal. Chemists Soc. 14th Ed. 26.014-26.018. Arlington, VA. (1984)

[b]Modified methods developed by Virginia Division of Consolidated Laboratory Services.

Table 2. Summary of the Monthly Analyses of Total Aflatoxin Contamination in Corn Meal during the Years 1983-1986[a].

1983

Month[b]	N[c]	NONE DETECTED[d]	<20 ppb[e]	21-100 ppb[f]	101-200 ppb[g]
JAN	53	43/81.1%	10/18.9%	NR[h]	NR[h]
FEB	25	20/80.0%	5/20.0%	NR	NR
MARCH	40	30/75.0%	10/25.0%	NR	NR
APRIL	23	17/73.9%	6/26.1%	NR	NR
MAY	17	11/64.7%	6/35.3%	NR	NR
JUNE	20	19/95.0%	1/ 5.0%	NR	NR
JULY	19	17/89.5%	2/10.5%	NR	NR
AUG	23	18/78.3%	5/21.7%	NR	NR
SEPT	23	20/87.0%	3/13.0%	NR	NR
OCT	55	36/65.5%	19/34.5%	NR	NR
NOV	61	34/55.7%	15/24.6%	12/19.7%	NR
DEC	240	114/47.5%	108/45.0%	18/ 7.5%	NR
TOTAL[i]	599	379/63.3%	190/31.7%	30/ 5.0%	NR

1984

Month[b]	N[c]	NONE DETECTED[d]	<20 ppb[e]	21-100 ppb[f]	101-200 ppb[g]
JAN	28	16/51.7%	12/42.9%	NR	NR
FEB	57	31/54.4%	22/38.6%	4/ 7.0%	NR
MARCH	48	13/27.1%	23/47.9%	12/25.0%	NR
APRIL	35	15/42.9%	17/48.6%	3/ 8.6%	NR
MAY	18	12/66.7%	6/33.3%	NR	NR
JUNE	21	15/71.4%	6/28.6%	NR	NR
JULY	26	15/57.7%	9/34.6%	2/ 7.7%	NR
AUG	61	19/31.1%	19/31.1%	23/37.7%	NR
SEPT	38	9/23.7%	27/71.1%	2/ 5.3%	NR
OCT	21	4/19.0%	17/81.0%	NR	NR
NOV	32	14/43.8%	18/56.3%	NR	NR
DEC	6	4/66.7%	2/33.3%	NR	NR
TOTAL[i]	391	167/42.7%	178/45.5%	46/11.8%	NR

1985

Month[b]	N[c]	NONE DETECTED[d]	<20 ppb[e]	21-100 ppb[f]	101-200 ppb[g]
JAN	30	16/53.3%	9/30.0%	5/16.7%	NR
FEB	10	9/90.0%	1/10.0%	NR	NR
MARCH	15	13/86.7%	1/ 6.7%	1/ 6.7%	NR
APRIL	21	17/81.0%	4/19.0%	NR	NR
MAY	28	5/17.9%	4/14.3%	19/67.9%	NR
JUNE	31	17/54.8%	14/45.2%	NR	NR
JULY	22	17/77.3%	5/22.7%	NR	NR
AUG	17	14/82.4%	3/17.6%	NR	NR
SEPT	18	17/94.4%	1/ 5.6%	NR	NR
OCT	24	18/75.0%	6/25.0%	NR	NR
NOV	23	17/73.9%	4/17.4%	2/ 8.7%	NR
DEC	29	10/34.5%	11/37.9%	8/27.6%	NR
TOTAL[i]	268	170/63.4%	63/23.5%	35/13.1%	NR

1986

Month[b]	N[c]	NONE DETECTED[d]	<20 ppb[e]	21-100 ppb[f]	101-200 ppb[g]
JAN	44	25/56.8%	15/34.1%	4/ 9.1%	NR
FEB	29	15/51.7%	14/48.3%	NR	NR
MARCH	13	6/46.2%	7/53.8%	NR	NR
APRIL	8	7/87.5%	1/12.5%	NR	NR
MAY	19	13/68.4%	6/31.6%	NR	NR
JUNE	29	18/62.1%	11/37.9%	NR	NR
JULY	23	12/52.2%	11/47.8%	NR	NR
AUG	39	25/64.1%	14/35.9%	NR	NR
SEPT	25	11/44.0%	14/56.0%	NR	NR
OCT	34	21/61.8%	13/38.2%	NR	NR
NOV	63	27/42.9%	27/42.9%	9/14.3%	NR
DEC	62	26/41.9%	26/41.9%	8/12.9%	2/ 3.2%
TOTAL[i]	388	206/53.1%	159/41.0%	21/ 5.4%	2/ 0.5%

[a]Analyses were conducted by the Food Laboratory of the Virginia Division of Consolidated Laboratory Services. The data presented herein are the results of AOAC approved methods for HPLC analysis of aflatoxins.
[b]Month in which the test was conducted.
[c]Number of samples examined.

(cont.)

Table 3 lists the 16 months in which corn meal testing yielded AFTs at levels greater than 20 ppb. The percentage of the total number samples having levels of AFT greater than 20 ppb and its corresponding month and year are ranked. The number of months with AFT levels greater than 20 ppb, for the four years studied is as follows: 1983, two months; 1984, six months; 1985, five months; 1986, three months. The highest percentage of AFT contamination, greater than 20 ppb, occurred in May, 1985 when 19, or 67.9%, of the samples tested were contaminated. The highest number of samples with AFT levels greater than 20 ppb occurred in August, 1984 when 23, or 37.7%, of the samples had AFTs greater than 20 ppb. Based on the USFDA's guidelines for acceptable levels of AFTs in human food (less than or equal to 20 ppb), 1985 reported the highest percentage of corn meal unfit for human consumption (13.1%) followed by 1984 (11.8%), 1986 (5.9%), and 1983 (5.0%) (Table 4).

Aflatoxins continue to be important human and animal health concerns. The levels found in corn meal samples during this study suggest that human exposure to AFTs at levels above the USFDA acceptable limit of 20 ppb may have occurred. This demonstrates the need for continued testing for AFTs and continued efforts on the part of regulatory agencies and the industry as well as the work by researchers to develop a control measures for food products which prohibits or reduces AFT levels without the treatment itself posing a additional risk.

The extent of AFT contamination in corn meal is not surprising when previous literature concerned with AFT contamination of grain corn in Virginia is consulted. Llewellyn and Katzen reported in 1977, that 22.1% of Virginia's grain corn submitted to the Agricultural Stabilization and Conservation Service of the United States Department of Agriculture (ASCS/USDA) had AFTs at Levels above that considered acceptable for human consumption (greater than 20 ppb). Mooney et al. (1990) reported that 58.5% of the ASCS/USDA submitted samples in 1986 had AFTs at levels greater than 20 ppb. These two citations are among a growing list of publications documenting AFT contamination in corn (Calvert et al., 1978, Lillehoj et al., 1978, Davis et al., 1986).

Table 2 (cont.)

[d] Number of samples/percentage of the total having undetectable levels (< 2.0 ppb) of total aflatoxins.

[e] Number of samples/percentage of the total having less than or equal to 20 ppb of total aflatoxins.

[f] Number of samples/percentage of the total having between 21 and 100 ppb of total aflatoxins.

[g] Number of samples/percentage of the total having between 101 and 200 ppb of total aflatoxins.

[h] NR, None reported.

[i] Cumulative data for the respective year.

Table 3. Ranking of Months With Samples Having Greater Than 20 ppb AFT.

Year	Month	% Greater Than 20 ppb[a]	Rank
1985	May	67.9%	1
1984	Aug	37.7%	2
1985	Dec	27.6%	3
1984	March	25.0%	4
1983	Nov	19.7%	5
1985	Jan	16.7%	6
1986	Nov	14.3%	7
1986	Dec	12.9%	8
1986	Jan	9.1%	9
1985	Nov	8.7%	10
1984	April	8.6%	11
1984	July	7.7%	12
1983	Dec	7.5%	13
1984	Feb	7.0%	14
1985	March	6.7%	15
1984	Sept	5.3%	16

[a]Percentage of corn meal samples tested having equal to or greater than 20 ppb of aflatoxins.

Table 4. Ranking of the Percentage of Aflatoxin (AFT) contamination equal to or greater than 20 ppb for the Years 1983-1986.

Year	% Greater Than 20 ppb	Rank
1985	13.1%	1
1984	11.8%	2
1986	5.9%	3
1983	5.0	4

It is the hope of the authors that a chronological and a historical documentation of AFT contamination in Virginia's agricultural products will not only serve as a record, but result in an increased awareness of the potential health hazards posed by aflatoxins.

SUMMARY

During the four years examined in this study, it was evident that aflatoxin contamination was carried through the milling and other processing treatments of grain corn. Of the 1646 samples tested, 722 or 43.8% showed detectable levels of AFTs. Also, 134, or 8.1% showed AFT occurrence in samples at levels greater than the USFDA maximum for human consumption of 20 ppb. Of the 599 samples tested in 1983, 220 or 36.7% showed detectable levels of aflatoxin (AFTs). Of the 391 samples tested in 1984, 224 or 57.3% had detectable levels of AFTs. Of the 268 samples tested in 1985, 98 or 36.6% had detectable levels of AFTs. Of the 388 samples tested in 1986, 180 or 46.4% had detectable levels of AFTs. For the four years examined, 1984 showed the highest extent of contamination with 57.3% of the samples tested reporting detectable levels of AFTs while 1985 had the highest percentage of samples above the USFDA's acceptable limit of 20 ppb, 13.1%. Despite the high percentage of detectable AFTs in 1984, 1985 shows the most severe contamination because of the higher percentage of samples tested that year that were unfit for human consumption. These levels paralleled, as expected, AFT levels reported for corn in the state.

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Soil Populations of *Aspergillus flavus* Conidia and Sclerotia

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INTRODUCTION

Many fungi of economic importance produce both conidia and sclerotia at some point during their life cycle. Each of these structures are produced to enhance the survival of the fungi during adverse environmental conditions (Coley-Smith and Cooke, 1971).

Conidia and sclerotia are non-motile, vegetative structures produced by many species of fungi, including Aspergillus flavus. Sclerotia are thick-walled structures consisting of a mass of fungal tissue. These structures are highly resistant to unfavorable conditions and may remain dormant for long periods of time. Upon the return of favorable growing conditions, the sclerotia germinate to continue the life cycle (Alexopolous and Mims, 1979; Moore-Landecker, 1972). Conidia are thin-walled, non-sexual spores that are borne terminally and exogenously on a conidiophore. Conidia are deciduous at maturity.

An understanding of the importance of sclerotia and conidia in the life cycle of A. flavus is important, especially as related to the agricultural importance of this organism. Many strains of A. flavus produce the highly carcinogenic mycotoxin, aflatoxin (Wogan, 1966). Aflatoxin has been shown to be toxic and carcinogenic to a wide variety of animals (Wogan and Newberne, 1967).

Since sclerotia may serve an important function in the life cycle of A. flavus, it is important to understand how this structure affects the long-term survival of this organism in the soil. Wicklow et al. (1982), have suggested that sclerotia represent a source of overwintering inoculum for A. flavus in field soils in which corn (Zea mays L.) is grown. Sclerotia can germinate the following spring and summer to continue the life cycle (Wicklow and Donahue, 1984; Wicklow and Wilson, 1986).

In the present study, we examined the survival of both conidia and sclerotia in soil. The differential population response of each of these

structures was followed to determine which structure was most important to the short and long-term survival of A. flavus.

MATERIALS AND METHODS

Replicate plots (1 by 1 m) were established at the University of Maryland Forage Research Farm in Clarksville, MD. The soil in this area is a silt loam. The area had been in continuous corn production for the past ten years. The soil contained no indigenous A. flavus. Plots were inoculated in October 1987 with either conidia or sclerotia of the following strains of A. flavus:

NRRL 6540 - conidia and sclerotia production, aflatoxin positive.

NRRL 6541 - conidia and sclerotia production, aflatoxin negative.

ATCC 15546 - conidia production only, aflatoxin production.

The treatments included, conidia of NRRL 6540, conidia of NRRL 6541, conidia of ATCC 15546, and sclerotia of NRRL 6540. To inoculate the plots, 3 kg soil was removed from each plot, dried and sieved. To this soil, 20 g ground corn stover was added. The purpose of the corn stover was to simulate the plowing down of infected corn into the soil. Each treatment was then inoculated with 5×10^{10} colony forming units (CFU) of conidia or 2×10^{3} sclerotia as described above. Soil and inoculum were thoroughly mixed. The inoculated soil were evenly spread over the surface of the original plots.

Soil samples were collected immediately after inoculation and every month for the first six months. Thereafter, soil samples were collected every two months. To collect the soil samples, a 2.0 cm auger was inserted into the soil to a depth of 15 cm. Three cores were collected per plot and the cores were composited into a single sample. After mixing of the composites, 10 g of soil was added to 95 ml sterile phosphate buffer (pH 7.6) and serially diluted to 10^{-6}. The dilutions were then plated onto a Botran-amended medium which is partially selective for A. flavus (Bell and Crawford, 1967). All plates were incubated at 25°C for 14 days. Each plate was then scored for viable A. flavus.

The number of sclerotia was determined by adding 10 g soil to 100 ml water. The mixture was shaken for a period of 5 min. The mixture was poured through No. 18 (1.0 mm) and No. 60 (250 μm) sieves. Sclerotia, which range in size from 260 to 680 μm, were retained on the No. 60 sieve. To further purify the sclerotia retained on the sieve, the contents were placed in a beaker of water and the buoyant debris carefully decanted. The sediment, consisting primarily of sclerotia, quartz, and other non-A. flavus sclerotia was transferred to 50 ml of 5 M sucrose in a centrifuge

tube. The mixture was shaken for 2 min. and then centrifuged at 3,500 rpm for 5 min. The supernatant containing the sclerotia was poured onto a filter paper under vacuum. The sclerotia were then hand picked with forceps from the small amount of remaining debris. At this point, the sclerotia were surface sterilized in sodium hypochlorite (0.25%) for 2 min. The sclerotia were rinsed two times in sterile, distilled water and transferred to the surface of the Botran-amended medium (Wicklow, 1987). All plates were incubated at 25°C for 3 days, after which viable sclerotia were counted.

RESULTS

Populations of A. flavus generally declined for the duration of the study (Figure 1). The population immediately after inoculation of the soil averaged 5 x 10^4 CFU per g soil, except where sclerotia were the only inoculum added to the soil. After 18 months of incubation, the population had been reduced approximately 10-fold. The only exception to the steady decline occurred in March 1988 when soil temperatures were increasing and an abundance of organic substrates were available to support the proliferation of the fungi.

No difference in survival was noted between A. flavus strains NRRL 6540 and 6541, the strains that produce and do not produce aflatoxin, respectively. Soil populations of each of these strains were very similar throughout the study. This observation suggests that the ability to produce aflatoxin has little effect on the survival of A. flavus in soil.

There was a large difference in soil populations depending upon whether conidia or sclerotia were inoculated into the soil. As can be seen, for strain NRRL 6540, the population in soil inoculated with sclerotia was nearly 10 to 20-fold less than soil that was inoculated with conidia. While the population was significantly lower than when conidia were inoculated into the soil, the presence of sclerotia in soil indicates that the sclerotia were rapidly germinating to release additional propagules into the soil. This observation was especially evident during April and May 1988 when the soil environment was ideal for sclerotia germination and growth. After this time, as the soil temperature increased and moisture became limiting, the soil population gradually declined.

The lowest soil population at the conclusion of the study was observed for strain ATCC 15546, the strain that did not produce sclerotia. Populations very rapidly declined from initial inoculation levels and exhibited the lowest population level of any of the treatments by the end

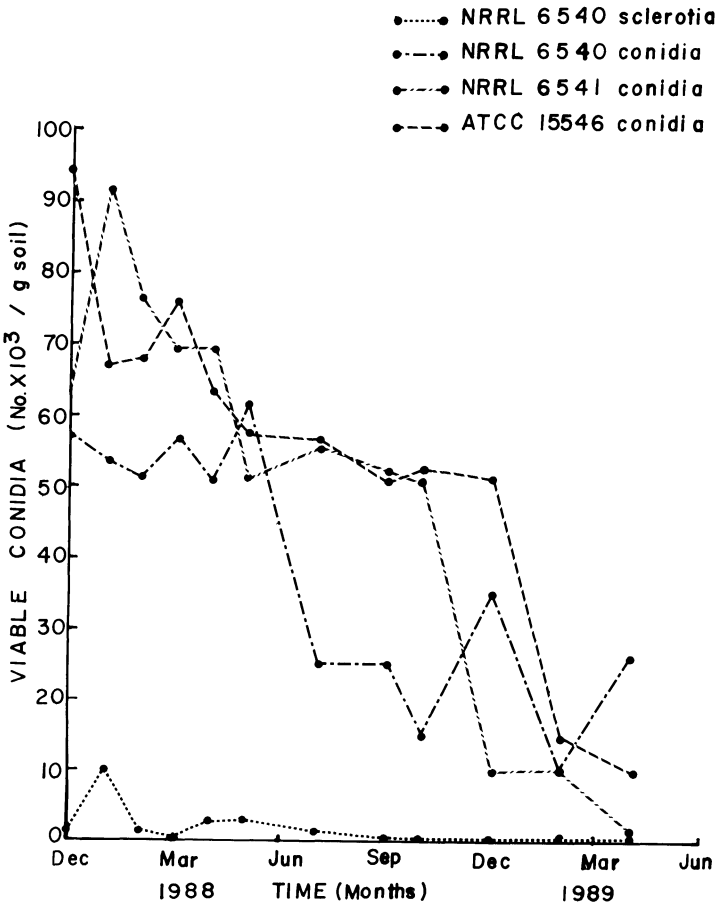

Figure 1. Number of Conidia in Soil Inoculated with Aspergillus flavus.

of the study. This observation further confirms that sclerotia are important for the long-term survival of A. flavus, especially during the winter months.

The number of sclerotia in soil over the duration of the study gradually declined (Figure 2). Generally, only one or two sclerotia per g soil were observed at any of the sampling dates. The presence of sclerotia in soil, however, where no sclerotia were originally added, indicated that sclerotia were being produced in the soil.

Strain ATTC 15546, which does not produce sclerotia, exhibited very low, yet detectable numbers of sclerotia during the first year of incubation in soil. There are several possible reasons why sclerotia could have been observed in this plot. First, cross contamination from adjacent plots could have provided spores from strains that produced sclerotia. While possible, this explanation is unlikely since each of the plots was surrounded by a wooden berm. It is also possible that sclerotia from fungi other than A. flavus could have been incorrectly identified. This explanation would be especially plausible if the surface of the sclerotia was not completely surface sterilized. Finally, it is possible that strain ATCC 15546, while not producing sclerotia on artificial media, does produce sclerotia in the soil. This observation will be further examined in the future.

Inoculation of sclerotia directly into the soil demonstrated that these structures degraded very slowly over time. After 18 months of incubation in the soil, the number of sclerotia declined from 53 to 20 per g soil. Numbers of sclerotia were much higher than any of the plots to which no sclerotia were added.

DISCUSSION

The suggestion by Wicklow et al. (1982) that sclerotia of A. flavus are an important source of overwintering inoculum in soil for cereal crops has been substantiated by the current study. Numbers of sclerotia in soil, while declining over time, were sufficient to ensure that adequate inoculum was present in the spring of each year to continue the infection cycle.

Wicklow (1987) first reported the occurrence of A. flavus sclerotia in standing corn and in ears lying on the soil surface. They further observed that sclerotia viability was substantially reduced in ears lying on the ground. This observation is supported by our results in that approximately 40% of the sclerotia isolated from soil after one year of incubation were not viable.

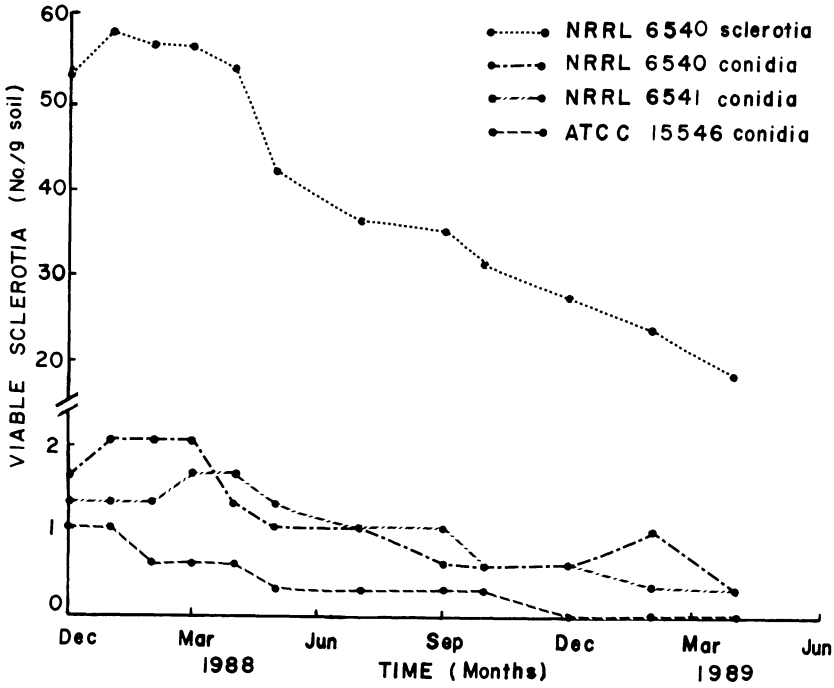

Figure 2. Number of Viable Sclerotia in Soil Innoculated with Aspergillus flavus.

Our data also provides evidence that sclerotia are not the only structure capable of providing a means of survival throughout the year. Conidia were shown to be capable of surviving in soil for a substantial period of time. Despite the rapid decline in numbers of these structures in the soil (90% decline during the entire study) levels were sufficient to be an important source of inoculum during the spring and summer of the following season.

In conclusion, this study shows that both conidia and sclerotia are important for the survival of A. flavus in soil. While conidial numbers declined more rapidly than sclerotia, levels of each were sufficient to infect the subsequent years corn crop. The current study, as well as other recent reports, indicate that the soil is an important, and probably the primary, source of inoculum for the infection of corn by A. flavus.

SUMMARY

The mycotoxigenic fungi, Aspergillus flavus, overwinters in the field as either conidia or sclerotia. Studies were conducted to determine which overwintering structure was most important for survival. Replicate field plots were established on a silty loam soil in continuous corn production for the previous ten years. Plots were inoculated with the following treatments: conidia of A. flavus strain 6540, sclerotia of A. flavus strain 6540, conidia of A. flavus strain 6541, and conidia of A. flavus strain 15546. Soil samples were collected monthly and the number of conidia and sclerotia determined by selective plating and a sieving/decanting procedure, respectively. Soil samples were collected and assayed for a two-year period. Results indicate that both sclerotia and conidia numbers in soil declined over time. During the two year study, the number of viable sclerotia declined by 60% while the number of viable conidia declined by 90%. Numbers of both structures observed in the spring of each year were sufficient to function as primary inoculation for the subsequent years' corn crop. These results therefore indicate that conidia may be important structures for survival over a single winter season, while sclerotia may provide a means of survival over a several year period.

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Quantifying Naturally-Occurrrng Macrocyclic Trichothecene Toxins

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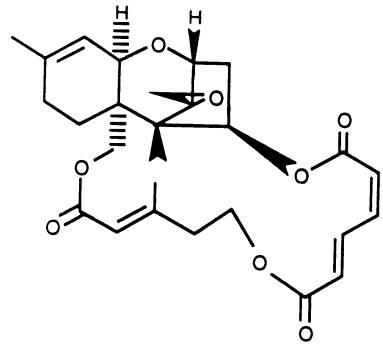
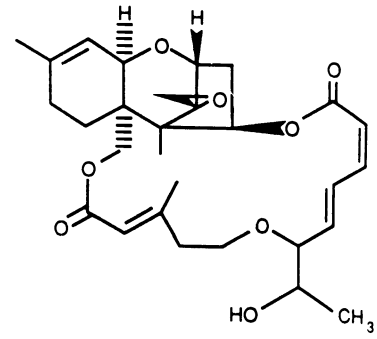
INTRODUCTION

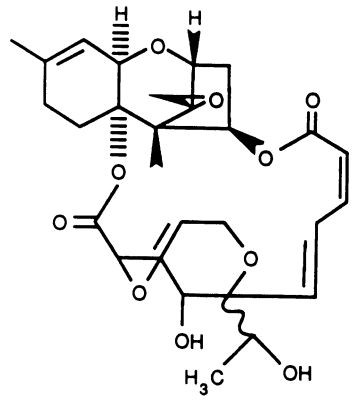
Macrocyclic trichothecene mycotoxins (Figure 1) are toxic metabolites produced by the fungi, Stachybotrys and Myrothecium (Bambarg and Strong, 1972; Jarvis et al., 1980). These fungi are considered both causative agents of stachybotryotoxicosis and plant pathogens. Various reports have appeared concerning macrocyclic trichothecene-induced mycotoxicosis and stachybotryotoxicosis (Adnrassy et al., 1980; Danko, 1976; Harrach et al., 1983; Schneider et al., 1979). These diseases have occurred in various regions of the world, e.g., South Africa (Schneider et al., 1979), Finnland (Hintikka, 1977), the Soviet Union (Yuskiv, 1968), and Hungary (Danko, 1976; Harrach et al., 1983). In addition, fungal strains which produce macrocyclic trichothecenes have been detected in Poland, East Germany, Hungary, Czechoslovakia, India, and Egypt. Therefore, the macrocyclic trichothecenes appear to be widely distributed.



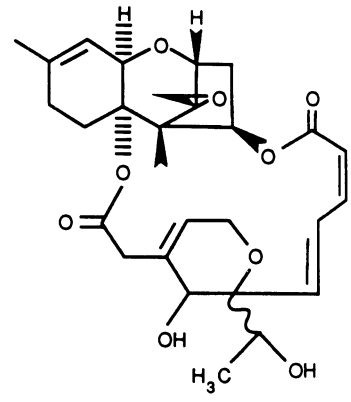
VERRUCARIN J



RORIDIN E



SATRATOXIN G



SATRATOXIN H

Figure 1. Structures of Some Macrocyclic Trichothecenes.

At present, only one analytical procedure for the quantification of the macrocyclic trichothecenes has been reported (Stack and Eppley, 1980). However, this procedure appears to be best suited to certain cereals where Stachybotrys atra may be overgrown by other fungi. In contrast, only a few fungi are capable of metabolizing cellulose, and this appears to be the primary reason for its dominance on this substrate. Thus, an analytical method and a brine shrimp bioassay (Eppley, 1974) are described which allow for both the qualitative and quantitative evaluation of satratoxin and verrucarin straw samples.

MATERIALS AND METHODS

Preparation of Authentic Macrocyclic Trichothecenes

Both satratoxins H and G were prepared in our laboratory (Bata et al., 1985), and their purities were established by mass and NMR spectroscopic methods. The verrucarin J used in these procedures was a gift from Dr. Tamm (University of Basel, Switzerland), and the roridin E was provided by Dr. Jarvis (University of Maryland, USA).

Sample Extraction and Clean-up Procedure

The straw samples were cut into 1 cm pieces, 50 g of each sample were placed in Erlenmeyer flasks, and individual samples were extracted with 300 ml of methanol for four hours. Two of these methanolic extracts were combined and evaporated to 50 ml. Next, 20 ml H_2O were added to the extract which was subsequently washed three times with 30 ml petroleum ether each time. The toxin-containing methanolic phase was evaporated within a water bath and the residual water was extracted three times with 10 ml ethyl acetate each time. The resultant ethyl acetate phase was evaporated to dryness with the residue being dissolved in both 1 ml methanol and 1 ml H_2O. This mixture was passed through a Sep-Pack C_{18} cartridge (Waters Associates Inc., Milford) which was then washed with 2 ml methanol-water (1:1). The toxins were eluted with 3 ml methanol-H_2O (8:2) from the cartridge which was regenerated with 5 ml ethanol and equilibrated with 5 ml methanol-H_2O (1:1) prior to re-constitution. The toxin-containing eluent was evaporated to dryness via a N_2 stream at 80°C and the residue was re-constituted in 100 µl methanol.

Thin Layer Chromatography (TLC)

Following spotting of the methanolic extracts together with authentic standards onto Kiesel gel 60, 10 x 10 cm HPTLC (Merck, Darmstadt, FRG) plates were developed in chloroform-isopropanol (44 + 6, v/v). The

separated compounds were detected by either UV light (254 nm) or by spraying the plates with 1% 4-(p-nitrobenzyl pyridine) solution followed by heating at 150°C for 30 min. and re-spraying with 10% tetraethylene pentamine reagent (Grob et al., 1981). The macrocyclic trichothecenes appeared as bluish-violet spots upon a white background.

High Performance Liquid Chromatography (HPLC)

The HPLC separations were performed with a Waters instrument on a 25.0 cm x 4.6 mm C_{18} poligosyl 60 D column (Macherey-Magel, Dwen, FRG). The gradient mode of application was employed with eluent A being methanol-H_2O (20:80, v/v) and eluent B methanol-H_2O (80:20, v/v). The running time was 20 min. Compounds which eluted with the same retention time as the authentic standards were collected and then evaporated to dryness under a N_2 stream.

Gas Liquid Chromatography (GLC)

Fifty µl of 0.5 N sodium methoxide reagent were added to the residue resulting from evaporation. Then, the mixture was placed into a block thermostated at 60°C for 15 min when 60 µl methanol containing 0.5 N HCl were added to the mixture to neutralize the base. Then, the mixture was evaporated to dryness with the subsequent addition of 100 µl BSTFA reagent. Next the mixture was again placed into a thermostat block at 60°C for 15 min. To investigate the bis-trimethylsilys ether derivative of verrucarol, a glass capillary tube moistened with SE stationary phase was employed (10.00 mm x 0.30 mm i.d.). The column temperature was 160-220°C, 4°C min^{-1} with a split ratio of 1:10.

RESULTS

Chromatography

Following TLC, the R_f values of satratoxins G and H as well as verrucarol were 0.53, 0.43, and 0.30, respectively. The sensitivity of the TLC method was 0.3 mg kg^{-1} for the quantification of verrucarol and 0.2 mg kg^{-1} for satratoxins G and H, respectively.

The sensitivity of the GLC method was 0.05 µg kg^{-1} for verrucarol while the sensitivity of HPLC was 0.01 mg kg^{-1} for satratoxins G and H and verrucarin J but 0.015 mg kg^{-1} for roridin E. Table 1 presents some data for field samples utilizing the three step procedure.

Quantitation and Recovery

To achieve quantitation, a calibration curve utilizing the appropriate authentic standard was constructed. To obtain a linear

Table 1. Natural Occurrence of Macrocyclic Trichothecenes in Field Samples[a].

Area of Sample Collection	Substrate Source	Satratoxin G (mg kg^{-1})	Satratoxin H (mg kg^{-1})	Verrucarin J (mg kg^{-1})
Budapest I	Straw	0.020	0.075	0.035
Budapest II	Straw	0.010	0.050	0.025
Vaja I	Straw	0.060	0.140	0.085
Kecskemet	Straw	0.020	0.065	0.030
Puszta	Hay	0.015	0.045	0.020
Vaja II	Straw	0.095	0.080	0.040
Lajosmizse I	Straw	0.050	0.070	0.035
Lajosmizse II	Straw	0.070	0.080	0.025
Csongrad I	Straw	0.025	0.040	0.010
Csongrad II	Straw	0.090	0.075	0.040
Budapest III	Straw	0.070	0.080	0.030
Budapest IV	Straw	0.080	0.140	0.650
Kecskemet II	Straw	0.120	0.110	0.850
Kecskemet III	Straw	0.157	0.185	0.112

[a]Data are the results of two parallel experiments.

relation between toxin amount and peak area, different quantities (10, 20, 50, 75, and 100 mg) of macrocyclic trichothecene toxins were injected into both gas and high pressure liquid chromatographs.

To assess recoveries, 5 μg satratoxins G and H as well as verrucarin J, respectively, were added to the samples in "spiking experiments" of which there were nine. The recoveries were 78, 72, and 68% for satratoxins H, G, as well as verrucarin J, respectively.

DISCUSSION

The most sensitive method (Stack et al., 1980) used for the determination of trichothecene macrocyclic toxins permits the quantification of those toxins to 0.20 mg kg^{-1}. Realizing that in many cases a concentration of 0.05-0.10 mg kg^{-1} may cause serious disease in animal husbandry, a more sensitive analytical method is needed. An advantage of the analytical method proposed here is that after both TLC and GLC it is possible to classify the sample into three categories: non, slightly, and highly toxic. An absence of verrucarol indicates non-toxicity and that HPLC investigation is not needed. Naturally for the determination of verrucarol, the GLC method is preferred rather than TLC.

Finally, for the regulation of the HPLC results, a second trans-methylation can be utilized which enables confirmation of the trichothecene skeleton employing authentic standards.

SUMMARY

Macrocyclic trichothecene mycotoxins are toxic metabolites produced by the fungi, Stachybotrys and Myrothecium (Jarvis et al., 1986). Although there is a variety of reports regarding macrocyclic trichothecene-induced mycotoxicoses, there are few available analytical procedures for both detecting and quantifying these trichothecenes. Here, we reported an improved three-step (TLC>GLC>HPLC) procedure for the quantification of satratoxins H and G, verrucarin J and roridin E. The recoveries for satratoxins H and G as well as verrucarin J, were 78.0, 17.2, and 68.0% respectively, following passage of 0.1 mg kg^{-1} of these toxins through the procedure.

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The Effects of Patulin on the Development of *Oncopeltus fasciatus*

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INTRODUCTION

Patulin (4-hydroxy-4H-furo(3,2) pyran-2(6H)-one) (Figure 1) has been shown to be a potent antibiotic with toxic effects in in vitro and in vivo studies (Kawasaki et al., 1972; Lindroth and von Wright, 1978; Oswald et al., 1978; Sorenson et al., 1985). Patulin is produced by many species of Aspergillus and Penicillium. Patulin was identified in 1942 by Wiesner, who observed its antibacterial activity when isolated from an Aspergillus clavatus culture. The compound was given the appropriate name "clavicin". Today both "clavicin" and "patulin" are commonly substituted. Patulin is considered a mycotoxin because of its toxicity and possible carcinogenic effects on higher life forms (Becci et al., 1981).

Patulin was used as an antibiotic, but was found to be quite toxic (Keilova-Rodova, 1949). Recently, studies have concentrated on patulin contamination of food products and animal feed. Patulin is found in many foods including apple juice produced from partially decayed apples and in germinated malt used for cattle feed (Jelinek, 1989).

The milkweed bug (Oncopeltus fasciatus) has served as an insect model in toxicologic studies so it was the objective of this study to determine patulin's toxicity via several parameters for this insect.

MATERIALS AND METHODS

Milkweed bugs were obtained from Carolina Biological Supply Company Incorporated, Burlington, NC. After receiving the colony, it was split into nine new cultures and each was allowed to mature and reproduce. Patulin was obtained from Sigma Chemical Company, St Louis, MO. Throughout the experiments

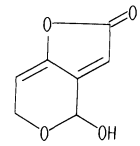

Figure 1. Structural Representation of Patulin.

Table 1. Mean Number of Days Until Maximum Number of Eggs Hatched as Related to Immersion Time.

Patulin Treatment[a]	Water Treatment[a] (Controls)	Immersion Time of Eggs (secs.)
-	8[b]	0[a]
8	7	20
6	9	40
9	7	80
10	7	160
6	8	320
5	8	640

[a]Mean elapsed time in days until maximum emergence occurred for ten egg groups.

[b]Unimmersed

the mean temperature was $70^{O}F \pm 10^{O}F$ and the photoperiod was 12 hours of light for all the treatments.

This study was divided into two phases. The first phase dealt with the effect of patulin exposure time on first instar nymph emergence. The milkweed bug eggs were exposed (by submergence) to 20.00 µg/ml patulin in distilled water for 0, 20, 40, 80, 160, 320, and 640 seconds. After exposure to patulin, all the eggs were placed in their respective group's Petri dish. The bottom of a smaller Petri dish was used to provide drinking water for emerged nymphs. Crushed sunflower seeds served as food. The number of newly emerged first instar nymphs was noted daily at 5:00 p.m. Appropriate control groups were established. There was an unimmersed group and groups immersed in sterile distilled water.

The second part of the study dealt with the effect of ingestion of varying concentrations of patulin on nymphs. Groups of 30 nymphs, 180 in total, were exposed to each of the following patulin levels in their drinking water, 20.00 µg/ml, 10.00 µg/ml, 5.00 µg/ml, 2.50 µg/ml, 1.25 µg/ml, and 0.00 µg/ml. The number of surviving insects at 5:30 p.m. each day was recorded.

RESULTS AND DISCUSSION

For the egg-treatment study, the number of days until no additional eggs hatched is listed in Table 1. Untreated, undisturbed eggs had eight out of ten nymphs to emerge. Table 2 shows the percent of hatched eggs having viable nymphs on day 14 of the study. Sixty three percent of the control eggs that hatched had surviving nymphs on day 14 of the study. Table 3 shows the mean accumulative lethality for the nymphs exposed to patulin in their drinking water.

There was very little effect on the eggs due to the time the eggs were exposed to distilled water. When these eggs were compared to the overall control eggs, that were not submerged, there was a slight difference. The difference indicated that the control eggs gave rise to a higher percentage of emerged young. This could be due to the handling of the eggs in the exposure process rather than the immersion itself. Increased times of distilled water submersion failed to decrease the nymph emergence rate. All of the patulin time treatments showed decreased hatching when compared with the identical water treatments. The patulin treatment decreased the hatching rate by 10%. This was irrespective of the time that they were exposed (dipped). Eggs exposed to patulin for 20, 40, 160, and 640 seconds seemed to contribute to the survival of the nymphs that did hatch. In the other two time exposures it did not seem to contribute to their survival. In all of the exposure times

Table 2. Percent of Hatched Eggs Having Viable Nymphs on Day Fourteen.

Nymphs Alive on Day Fourteen						
	Water Treated Eggs			Patulin Treated Eggs		
Treatment Time in Seconds	No. Alive	No. Eggs Hatching	% Hatch Alive	No. Alive	No. Eggs Hatching	% Hatch Alive
0[a]	2	9	22.2	--	--	--
0	6	9	66.6	--	--	--
0	7	7	100.0	--	--	--
X±SD	5±2.65		63			
20	6	6	100	5	6	83.3
20	3	4	75	6	6	100
20	3	5	60	3	5	60
X±SD	4±1.73		78	4.67±1.53		81
40	6	7	85.7	3	4	75
40	4	5	80	2	3	66.7
40	2	4	50	4	4	100
X±SD	4±2		72	3±1		81
80	6	6	100	4	4	100
80	8	8	100	5	6	83.3
80	4	4	100	4	4	100
X±SD	6±2		100	4.33±.58		94
160	4	6	66.7	5	5	100
160	3	4	75	6	6	100
160	3	4	75	5	5	100
X±SD	3.33±.58		72	5.33±.58		100

320	6	6	100	1	3	33.3
320	5	6	83.3	0	2	0
320	4	6	66.7	4	4	100
X±SD	5±1		83	1.67±2.08		44
640	5	6	83.3	3	3	100
640	4	5	80	6	6	100
640	4	4	100	3	4	75
X±SD	4.33±.58		88	4±1.73		92

[a]Received no immersion or treatment in water or patulin.

Table 3. Mean Accumulative Lethality Due to Nymphs Drinking Patulin[a].

Concentration of Patulin in Drinking Water (μg/ml)	Days of Treatment				
	3	6	9	12	15
0.00	1.7±1.2	4.5±1.4	7±1.4	7.5±2.1	8±2.8
1.25	1±1	1.7±1.3	4.5±0.7	6.5±0.7	7±1.4
2.50	0.7±0.6	2.7±0.6	3.7±0.6	4.7±0.6	5±1
5.00	1±1	2.7±3	5.3±3.1	6.7±3.2	6.7±3.2
10.00	0±0	2.7±2.1	5.3±1.2	8.3±1.5	8.7±1.5
20.00	0±0	1.3±1.5	5.7±1.5	7±1	7±1

[a]X±SD from triplicate groups having 10 animals each.

except 320 seconds, patulin treatment increased nymph survival when compared to the other egg groups not treated with patulin. A trend is evident that dipping the eggs in both water and patulin contributed to the survival of the nymphs. This phenomenon was also observed in another aspect of the study. This observation was that longer exposure times yielded more nymphs.

Patulin ingestion studies (Table 3) showed that at the various concentrations used, there was no significant effect on the nymphs. There was also observation during the experiment, that the controls were performing poorly. This poor control showing may have been due to temperature variations. Another possible reason for low control values might be that the patulin exposure actually contributed to nymph survival. The study also showed that at these concentrations, patulin is less toxic than expected when compared to the toxicity of other mycotoxins in the insect model (Chinnici et al., 1976; Llewellyn et al., 1977; Llewellyn and Chinnici, 1978; Sherertz et al., 1978). A test using higher concentrations of patulin would be limited due to its solubility. The eggs were more sensitive than the nymphs, but they too failed to produce conclusive results. Earlier developmental stages seem more sensitive to the effects of patulin than the nymphs.

In the timed exposure phase of the study there were two sets of results. These were the number of eggs that hatched and the number that survived after hatching. There were higher hatching rates in the egg sets dipped in water as compared to the eggs that were dipped in patulin. However, among the eggs that were dipped in patulin there was a higher nymph survival rate than in the eggs exposed to water. In the ingestion study, the results were inconclusive because of high control group mortality. It seemed that the patulin might have contributed to the survival of the nymphs.

The conclusions drawn form this two-part experiment are similar. In both parts, patulin seemed to contribute to the nymphs' survival. Another conclusion drawn was that any kind of exposure (water or patulin) decreased the hatch. Also from the ingestion study, it was shown that the concentrations of patulin used were not sufficient to be seriously toxic to the nymphs. In summary, neither milkweed bug eggs nor their nymphs are highly sensitive to patulin. Patulin does not seem to be highly toxic in this assay system.

The non-toxic effects of patulin cannot be generalized for all mycotoxins. Gaston and Llewellyn (1980) reported reported that the LD_{50} for aflatoxin B_1 in milkweed bugs (5μg/ml in drinking water) was reached at day 8 for females and at day 24 for males in the study. Llewellyn et al. (1988) also reported toxic responses in milkweed bugs exposed to aflatoxin B_1. In

general, the responses recorded in this study as compared to other insect studies reflect the wide variation of insect responses to compounds which mammals generally find toxic.

SUMMARY

The effects of patulin, an antibiotic and fungal toxin, on milkweed bug egg hatching and nymph viability was studied. Water and patulin-immersed eggs were treated at seven time periods ranging from 0 to 360 seconds. Increased patulin treatment time failed to substantially reduce hatching. All time groups treated with patulin did show slightly lower hatching percentages. Surviving nymphs from patulin-treated eggs had somewhat higher survival rates. Other nymphs drinking various concentrations of patulin also tended to have better survival levels than those receiving distilled water. It appears that patulin is not very toxic to the milkweed bug egg and nymphs and the eggs and nymphs may actually benefit from patulin exposure.

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SECTION II

PROCESSES OF WOOD DECAY AND DETERIORATION

PROCESSES OF WOOD DECAY AND DETERIORATION

INTRODUCTION

The wood preserving industry in the United States treats approximately 300 million cubic feet of wood annually. The industry is based essentially upon three major preservatives--creosote, pentachlorophenol, and inorganic arsenicals--which have been successfully developed over a long period of use. Unfortunately, these are broad-spectrum poisons that pose a serious threat to the environment. The problem is twofold; disposal of toxic waste and treated wood materials, and pollution of the environment by treated wood products in use. At present, no harmless substitutes for toxic chemicals are available. Thus there is an urgent need to generate information that will lead to the development of effective and environmentally safe treatments for protection of wood products against wood-destroying organisms.

One approach to making significant advances in new or improved treatments to control decay is to promote a better understanding of the biochemical pathways of the organisms involved in the wood-decay process. The physiology of fungi provides for fungal nutrition and reproduction despite dynamic defenses within the living tree and preformed protective materials of wood in service. If it is known how fungi colonize and and break down wood, then it is possible to "sabotage" their ability to decay wood. For example, if the wood-decay process requires the presence of a transition metal, it would be possible to immobilize the metal in the wood and prevent the fungus from obtaining it; this would stop the decay process. This method of control would be environmentally safe because it specifically interferes with the metabolism of the wood-decay fungus and would not endanger nontarget organisms. The papers included in this chapter discuss several different aspects of fungal degradation and biochemistry, which should aid in uncovering the Achille's heel in the

wood-decay process and which can guide the development of new or improved treating methods for the future.

Several papers address the interaction of decay fungi with wood using electron microscopy and immunological techniques to localize fungal degrading agents. A common morphological feature of wood-decay fungi is the presence of extracellular layers (hyphal sheaths), which are composed of extensive membrane structures and B-1,6-1,3 glucans. This matrix probably plays a key role in degradation, but its specific function is still unknown. Of interest, and probably very important to our understanding of the decay process, is that an isolate of a decay fungus unable to decay wood did produce an abnormal hyphal sheath and failed to form the glucan typical of degradative isolates. Decay was also probed by cytochemical and immunological probes. Transmission electron microscopic (TEM) immunolabelling of manganese-dependent peroxidase in white-rotted wood showed the presence of the enzyme in the degraded middle lamellae and also in degraded areas of the wood cell wall.

For digestion of wood cell wall constituents, enzymes must be released externally to the fungus. Thus, one method of preventing fungal attack of wood would be to inactivate the extracellular degrading enzymes in situ or prevent their release from fungal hyphae. However, wood-decay fungi usually produce small amounts of extracellular degrading enzymes, which makes study of these enzymes difficult. One study involved recombinant DNA technologies that can be possibly utilized to achieve overproduction and enhanced secretion of wood-degrading enzymes. Along this same theme, another paper reports that high levels of simple sugars retarded white-rot decay, most likely by repressing degrading enzymes. Thus fungal wood-decay may be inhibited by nonmetabolizable sugar analogs.

It is important to understand activities during the previsual stages of decay. Commonly occurring pioneer fungi were reported to accelerate the decay process by their activity during previsual stages of decay. Studies also report the rapid accumulation of ions in wood in the early stages of decay. A better understanding of ionization and nondecay pioneer fungi should lead to improved methods of detecting and preventing biodeterioration of wood.

The damage produced by sapstreak disease of sugar maple is reported. Although sapstreak does not affect wood strength the discoloration reduces the value of maple logs and lumber 25% to 75% and limits exportation. This disease may present a model system for investigating aspects of immunity in trees.

Although little is known about nitrogen metabolism in wood-decay

fungi, such metabolism plays the most important role of the nonstructural nutrients in wood. The very low amounts of nitrogen in wood indicate that wood-decay fungi have a very efficient mechanism of nitrogen metabolism and reuse. Polyamines are nitrogen compounds found in fungi that are essential for growth and development. The biosynthesis of polyamines in most organisms can proceed through one of two metabolic pathways. However, only one pathway has been reported to be the route of synthesis in fungi. This feature makes the pathway an idea target for specific regulation of polyamine production in wood-decay fungi. The result would be the specific control of decay through control of fungal growth and development. Enzyme-activated irreversible inhibitors of polyamine biosynthesis were used to study polyamine metabolism in a brown-rot fungus. Ornithine decarboxylase (ODC) was found to be an important enzyme in fungal development. Decay in wood was also inhibited by the ODC inhibitor difluormethlyornithine, suggesting that polyamine inhibitors should be further studied for their potential use as wood preservatives.

Another approach for environmentally safe wood protection is to exploit relationships that already exist in nature. Some organisms that grow in wood, including fungi and bacteria, do not cause decay and are antagonistic to wood-decay fungi. This type of activity is termed "biological control." One paper discussed the possibility of using bacteria for biological control of wood-attacking fungi. A mixture of six bacteria prevented decay and attack by blue stain and mold in pine.

In the future, wood protection will depend less on the use of broad spectrum metabolic poisons. With the tools of biotechnology for studying problems of wood degradation and protection, our understanding of decay processed should advance rapidly in the coming years. These studies will reveal new and exciting information on mechanisms of decay, which will allow us to interfere with essential biochemical processes and to develop highly active and sharply targeted preservatives with enhanced performance.

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Polyamine Biosynthesis in the Brown-Rot Fungus *Postia placenta*

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INTRODUCTION

Polyamines are aliphatic polycations that have been shown to be essential for optimal growth and development of a wide variety of living organisms (McCann et al., 1987). Ornithine decarboxylase (ODC) (EC 4.1.1.17) and arginine decarboxylase (ADC) (EC 4.1.1.19) control the rate-limiting steps responsible for biosynthesis of the polyamines putrescine, spermidine, and spermine. The enzymes ODC and ADC catalyze the decarboxylation of ornithine and arginine, respectively. Both reactions lead to the formation of the polyamine putrescine, the precursor of spermine and spermidine (McCann et al., 1987).

The biosynthesis of polyamines in bacteria (Tabor and Tabor, 1985) and plants (Slocum et al., 1984) can proceed through the ODC or the ADC pathway, with ADC apparently being the primary pathway in plants (Birecka et al., 1986). In contrast, the ODC pathway has been reported to be the route for synthesis in fungi that are pathogenic to plants (Rajam and Galston, 1985; Birecka et al., 1986; Rajam et al., 1986). This feature makes the ODC pathway an ideal target for controlling plant disease by regulation of polyamine production in the pathogen without harming the host plant.

The synthesis of rationally designed ODC and ADC inhibitors has become the logical approach to preventing polyamine synthesis. The inhibitors

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are enzyme specific and irreversible, acting at the catalytic site of the enzyme, which results in "suicide." The "suicide" enzyme inhibitors for ODC and ADC are DL-α-difluoromethylornithine (DFMO) (Metcalf et al., 1978) and DL-α-difluoromethylarginine (DFMA) (Kallio and McCann, 1981), respectively. They have recently gained prominence in studies of enzyme regulation, in the determination of the reaction steps in the polyamine pathway (Pegg, 1987), and in the unraveling of the physiological role of polyamines in cell functions (Marton and Morris, 1987).

Polyamines have not been studied in the fungi that degrade wood. The objectives of the present study were to use the brown-rot fungus Postia placenta (Fr.) M. Lars. et Lomb. (MAD-698) as a model (1) to determine the importance of polyamines on growth of a wood-decay fungus, (2) to determine the primary biosynthetic pathway in the fungus by employing a "suicide" enzyme inhibitor, and (3) to test the efficacy of using DFMO to prevent growth and development of the fungus in wood.

MATERIALS AND METHODS

Cultures of P. placenta were maintained on malt agar slants at 4°C. Mycelia from slant cultures were used to inoculate stock culture plates containing a basal medium and 1% cellobiose as the carbon source. The basal medium contained mineral salts and thiamine as previously described (Highley, 1973). Stock cultures were incubated at 27°C and 70% relative humidity (RH). Enzyme inhibitors were from Merrell-Dow (Cincinnati, Ohio)[2]. All other reagents were from Sigma, Baker, or Difco.

Inhibitor-containing medium was prepared by adding filter-sterilized DFMO or DFMA aqueous solution to sterile medium containing minerals, thiamine, and cellobiose as in the stock culture. The final concentration of DFMO and DFMA was 0.1 mM, 0.5 mM, 1.0 mM, or 5.0 mM. Culture medium with no inhibitor served as the control.

Because DFMO is an enzyme-activated, irreversible inhibitor of ODC, addition of ODC reaction products to the culture medium should reverse any mycelial growth retardation that is due to the presence of DFMO. To test for the reversal of DFMO inhibition, a separate experiment was conducted

[2]The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the U.S. Department of Agriculture of any product or service to the exclusion of others that may be suitable.

in which cellobiose-basal medium containing 1.0 mM DFMO was amended with 0.1 mM putrescine or 0.1 mM spermidine.

In all experiments, 20 ml of the medium was dispensed into 100 by 20 mm sterile Petri plates. A 10 mm plug taken from the advancing edge of stock culture plates was inverted in the center of each experimental plate. Three plates were prepared for each replicate, and experiments were repeated two times. Plates were incubated in the dark at 70% RH and 27°C. Diameters of colonies were measured 3, 6, 9, and 14 days after the plates were inoculated. Measurements include the 10-mm inoculum.

The standard method for testing wood preservatives by the American Society for Testing and Materials, method D1413-61 (ASTM, 1961), was used to test the efficacy of DFMO as a fungal growth inhibitor in wood. Briefly described, this method employed laboratory cultures in which a Southern pine "feeder strip" of wood inoculated with P. placenta was placed on autoclaved soil, forming a ground contact equivalent. The soil-block cultures were incubated for 3 weeks at 27°C in the dark. Prior to fungal inoculation on feeder strips, test blocks were conditioned to equilibrium moisture content at 27°C and 70% RH and weighed. Test blocks of Southern pine or sweetgum (2.5 by 2.5 by 1.0 cm) were soaked for 24 h in an aqueous solution of 0.1%, 1.0%, or 5.0% DFMO. Control blocks were soaked in sterile, distilled water. After a 10-week incubation at 27°C and 70% RH, the blocks were dried to equilibrium moisture content and weighed. The loss in weight of each block was used as an indication of the extent of fungal damage. Four replicates were used for each treatment. The average percentage of weight loss was calculated for each treatment.

RESULTS

The effect of DFMO on growth of P. placenta is given in Figure 1. Growth of the fungus was suppressed by the enzyme inhibitor, and inhibition of growth was concentration-dependent. For at least 6 days, mycelial growth on all DFMO-containing plates was inhibited. Mycelia grew only 1 mm from the inoculum after 14 days on 5 mM DFMO. When putrescine or spermidine was added to DFMO-containing medium, the suppression of growth was reversed (Figure 2).

Mycelial growth of P. placenta was suppressed when the arginine decarboxylase inhibitor DFMA was added to the medium (Figure 3). Mycelia in control plates grew in a typical radial pattern from the inoculum plug. However, mycelial growth in DFMA-containing plates was initiated on one side of the inoculum plug, away from the light source used in the

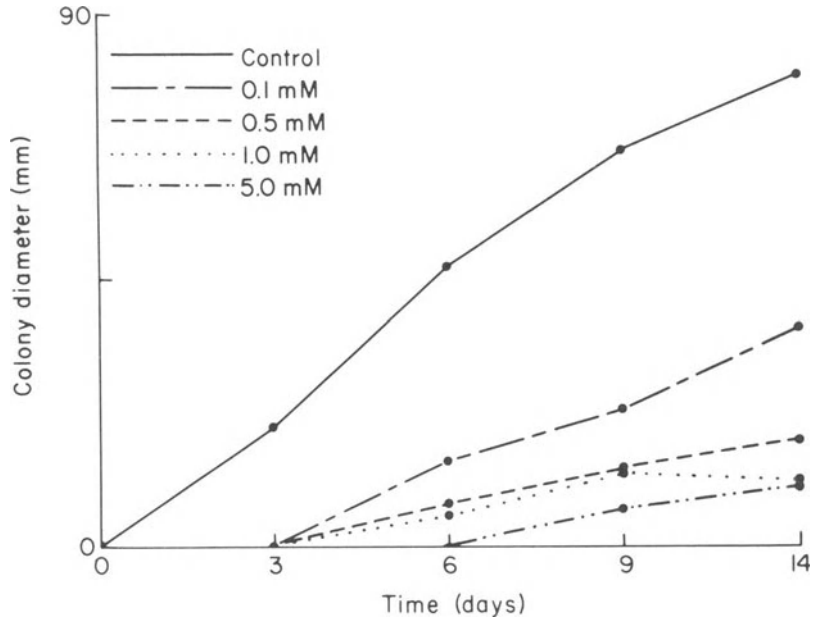

Figure 1. Effect of the 'Suicide' Inhibitor of Ornithine Decarboxylase, DFMO, on the Growth[a] of the Brown-rot Fungus, Poria placenta.

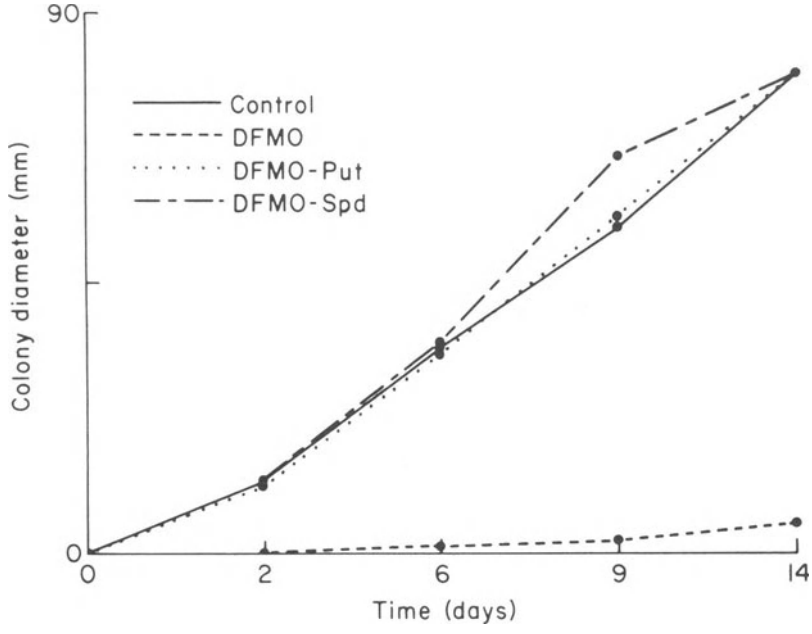

Figure 2. Reversal of DFMO Inhibition of Poria placenta Growth[a] by the Metabolic Products of Ornithine Decarboxylase, Putrescine and Spermidine.

incubation room for periodic maintenance of specimen. Measurements of colony growth were based on the total diameter of mycelial growth on one side of the plug. Growth was not suppressed as much with DFMA as with DFMO.

Southern pine and sweetgum blocks were resistant to decay by P. placenta after treatment with DFMO in aqueous solution (Figure 4). The inhibition of growth on wood was concentration-dependent. Mycelia were more visible on Southern pine (Figure 4A) than on sweetgum (Figure 4B).

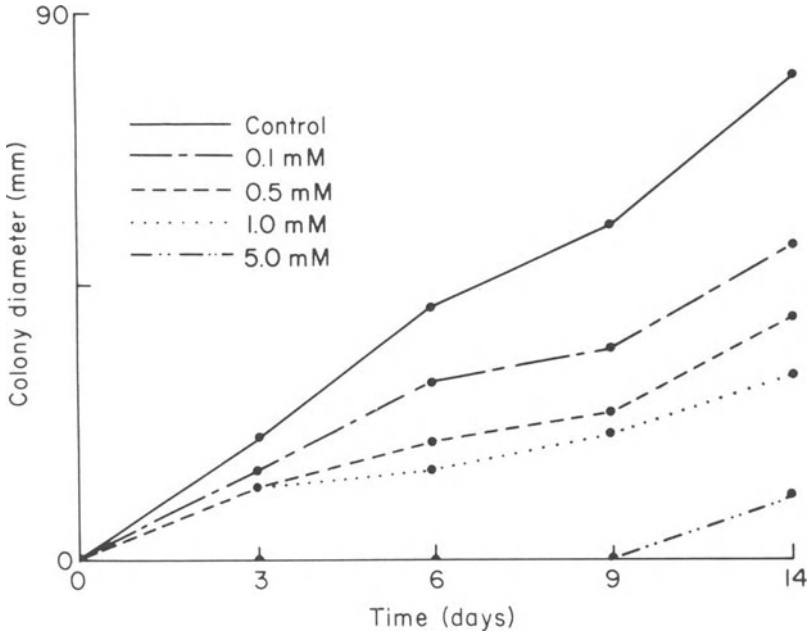

Figure 3. Effect of DFMA, "Suicide" Inhibitor of Arginine Decarboxylase, on Growth of Brown-Rot Fungus Poria placenta. Growth Expressed as Diameter of Mycelia in Millimeters.

The DFMO treatment decreased the percentage of weight loss of both wood species (Table 1). Untreated Southern pine and sweetgum inoculated with the fungus had weight losses of 55% and 64%, respectively. For Southern pine, the percentage of weight loss dropped from 39% with 1% DFMO to 7% with 5% DFMO; for sweetgum, the percentage of weight loss dropped from 46% with 1% DFMO to 4% with 5% DFMO.

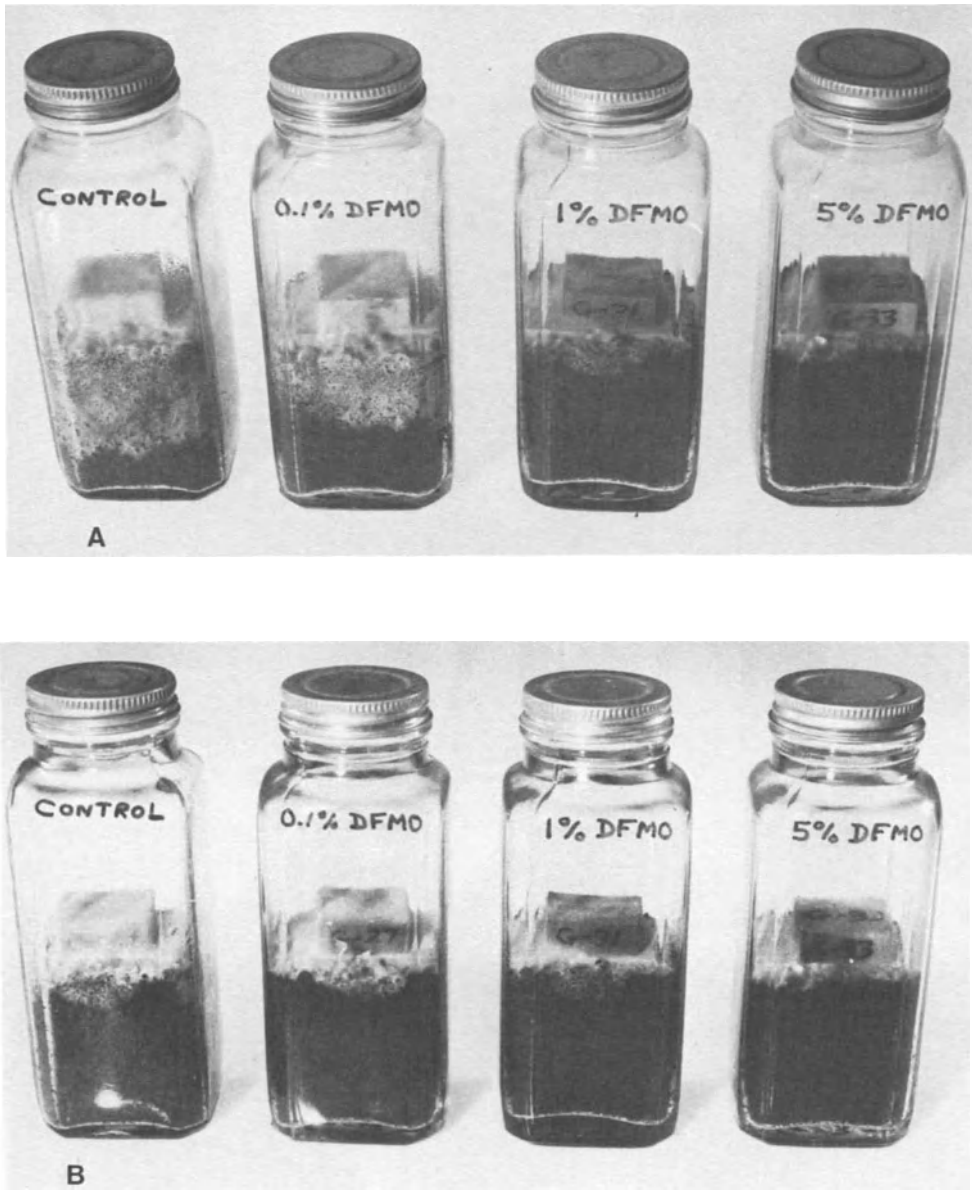

Figure 4. Standard Wood Blocks Exposed to Poria placenta; Pretreatment With DFMO; (a) None, (b) 0.1 mM, (c) 1.5 mM, (d) 1.0 mM, (e) 5.0 mM. A, Southern Pine; B, Sweetgum.

Table 1. Effect of the Ornithine Decarboxylase Inhibitor DFMO on Percentage of Weight Loss of Standard Wood Blocks Exposed to Poria placenta.

Concentration of DFMO (%)	Weight loss[a] (%)	
	Southern pine	Sweetgum
0 (control)	55	64
0.1	56	61
1.0	39	46
5.0	7	4

[a]At least four blocks per treatment in 10-week tests.

DISCUSSION

The enzyme-activated, irreversible inhibitor of ODC suppressed the in vitro mycelial growth of P. placenta, an indication that polyamines are essential for growth of the fungus. Growth was restored by addition of two products of the ODC metabolic pathway, putrescine and spermidine. Reversal of DFMO-induced growth inhibition indicates that the ODC pathway is probably the primary polyamine biosynthetic pathway in P. placenta. Biosynthesis of putrescine is reported to occur soley by the ODC pathway in those fungi tested to date (Tabor and Tabor, 1984). Our report is the first report of a polyamine biosynthetic pathway in wood-decay fungi.

Treatment with DFMO protected wood blocks against decay by the fungus, although total protection was not achieved. The concentration of inhibitor required to prevent decay would have to be greater than 5.0% in an aqueous solution. Several reasons could account for this result. The delivery of DFMO into the wood could have been incomplete, leaving areas where the fungus could grow. The insufficient adherence to the wood surface of DFMO in an aqueous solution could also have allowed fungal penetration and subsequent colonization. A different solvent system might enhance the usefulness of DFMO as a wood protectant. The rational design of wood protective chemicals should consider wetting agents and a fungicide delivery system in the formulation.

The *in vitro* growth of *P. placenta* was suppressed by DFMA, but less than the suppression by DFMO. The result is consistent with the reported DFMA effect on other fungi. In a study of the wilt fungus of tomatoes, *Virticillium dahliae*, DFMA was presumably converted to DFMO by arginase because activity of ADC was very low and could result from other aspects of arginine metabolism (Slocum and Galston, 1985). Arginase can convert DFMA to DFMO, and the arginase activity in *V. dahliae* was high enough to catalyze the conversion (Mussell et al., 1987). Growth was also retarded by DFMA as well as DFMO in 12 species of dermatophyte fungi (Boyle et al., 1988). The endogenous conversion of DFMA to DFMO was suggested by the authors.

Bacteria appear to be the only microorganism to have arginine decarboxylase activity (Bitonti and McCann, 1987). Growth suppression by DFMA in *P. placenta* may be due to a high arginase activity. If arginase activity is high in wood-decay fungi, conversion of arginine to ornithine could be another source for putrescine synthesis that would be subverted by use of DFMO.

Polyamine metabolism represents a major metabolic pathway in wood decay fungi that may serve as the "Achilles heel" for control of the destructive microorganisms. Enzyme-targeted inhibitors of polyamine synthesis represent a potential vehicle for this control. Until application formulas are designed, DFMO and DFMA may be limited to use as research tools in testing enzyme function and determining the role of polyamines in fungal physiology.

SUMMARY

Enzyme-activated irreversible inhibitors of polyamine biosynthesis were used to study polyamine metabolism in the brown-rot fungus *Postia placenta*. The effect of the enzyme-specific inhibitors for ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) were difluoromethylornithine (DFMO) and difluoromethylarginine (DFMA), respectively. The inhibitors suppressed *in vitro* mycelial growth, indicating that polyamines are essential for the fungus. Growth was retarded more by DFMO than by DFMA. Addition of the ODC pathway products, putrescine and spermidine, reversed the DFMO-induced inhibition. Growth in wood samples was also inhibited by DFMO in the ASTM standard wood-block test, suggesting that polyamine inhibitors should be further studied for their use as wood preservatives.

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Factors Associated with Decay Capacity of the Brown-Rot

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INTRODUCTION

Brown-rot basidiomycetes are a major cause of decay and decomposition of wood and wood products throughout the world. The rapid depolymerization of cellulose associated with brown rot results in serious strength losses of wood early in the decay process (Cowling, 1961); brown-rotters thus cause more damage in less time than the more prevalent white-rotters. Knowledge of the physiology and biochemistry of brown rot has lagged behind that of white rot. A better understanding of the mechanisms of brown rot could pinpoint specific metabolic pathways necessary for decay development and form the bases of future control strategies.

Strains that are unable to degrade wood efficiently are useful biological tools in the identification of metabolic pathways necessary for brown-rot development. Such a strain, designated ME20, has been described for Postia placenta (Fr.) M. Lars. & Lomb. (Micales and Highley, 1989; Micales et al., in press). This strain is monokaryotic and produces little change in the percent weight loss of wood. Isolate ME20 also exhibits an abnormal, appressed colony morphology (Micales and Highley, 1989). It produces carbohydrate-degrading enzymes, hydrogen peroxide, and oxalic acid (Micales and Highley, 1989), all thought to be important in the development of brown rot (Highley, 1987). Culture filtrates of ME20 do not seem to be as viscous as those produced by degradative strains of P. placenta (Micales and Highley, 1989).

[1]The Forest Products Laboratory is maintained in cooperation with the University of Wisconsin. This article was written and prepared by U.S. Government employees on official time, and it is therefore in the public domain and not subject to copyright.

Isolate ME20 superficially resembles certain strains of Schizophyllum commune Fr. that fail to produce aerial hyphae and do not secrete extracellular carbohydrates into culture media (Wessels and Niederpruem, 1967; Wessels et al., 1972). In S. commune, a basidiomycete with a bifactorial mating system (Raper and Miles, 1958), both traits have been associated with mutations in the A and B mating factors that relate directly to cell wall synthesis (Wessels and Niederpruem, 1967; Wessels, 1969). These strains produce higher concentrations of R-glucanase, a hydrolytic enzyme that degrades the alkali-insoluble glucan in the fungal cell wall. Certain morphological features, such as the appressed mycelial growth on agar (Wessels and Niederpreum, 1967) observed in ME20 and the S. commune isolates, may result from elevated levels of R-glucanase.

The objectives of this study are to determine whether (a) an appressed morphology on agar is associated with decreased decay ability; (b) differences exist in the quantities of extracellular carbohydrates produced by the nondegradative strain, ME20, and degradative strains of P. placenta; (c) the quantity of extracellular carbohydrate produced in culture can be correlated with decay ability and colony morphology; and (d) other physiological properties, such as mycelial growth, R-glucanase production, and sensitivity to selected fungitoxicants, can be correlated with extracellular carbohydrate production and decreased decay ability.

MATERIALS AND METHODS

Culture Maintenance and Determination of Decay Ability

All isolates are stored in the culture collection of the Center for Forest Mycology Research, USDA Forest Service, Forest Products Laboratory, Madison, Wisconsin. Collection information is presented in Table 1. Wood decay capabilities of P. placenta isolates were determined by the standard ASTM soil block method (ASTM, 1971) using southern pine (Pinus spp.) blocks (25.4 by 25.4 by 3.2 mm, the long axis parallel to the grain). Soil block bottles were incubated at 27°C and 70% relative humidity for 12 wks. At the end of this period, the percent weight loss of each block was determined.

Generation of Mutants

Mutants of P. placenta were formed by exposing hyphal fragments of a wild-type isolate, MAD698, to ultraviolet (UV) light. The fungus was initially grown in 25 ml 1% cellobiose in a basal salt solution (Highley, 1973) for 2 weeks. The liquid was aseptically removed and the mycelium

Table 1. Collection Data on Isolates of *Postia placenta* Fr.

Isolate number	Date of acquisition	Source	Additional information
MAD698 = ATCC11538	12/41	Yacht "America" decking *Pseudotsuga menziesii*	Dikaryotic
TRL2556	3/50	Mineshaft support, Transvaal, Africa	Dikaryotic
RLG3760R	7/63	*Betula alleghaniensis*, Newcomb, N.Y.	Dikaryotic
ME20	Unknown	Floor planking, Pleasant Hill, CA	Monokaryotic
L8035spA	9/57	Conifer log, Olympic Peninsula, WA	Monokaryotic test strain for A mating type
ME20 X L8035spA	3/87	Forest Products Laboratory	Dikaryotic; derived from cross of ME20 and L8035spA
UV27-5	7/88	Forest Products Laboratory	Mutants induced by exposure of MAD698 to ultraviolet light
UV28-18	7/88	Forest Products Laboratory	
UV32-7A	7/88	Forest Products Laboratory	
UV39-6A	7/88	Forest Products Laboratory	
UV39-6B	7/88	Forest Products Laboratory	
UV51-1B	7/88	Forest Products Laboratory	
UV51-1A	7/88	Forest Products Laboratory	
UV55-5	7/88	Forest Products Laboratory	
UV56-5A	7/88	Forest Products Laboratory	
UV59-1	7/88	Forest Products Laboratory	
UV64-4	7/88	Forest Products Laboratory	

macerated in a [2]Waring blender with 100 ml sterile distilled water and further diluted 1:500 with sterile distilled water. One-milliliter samples were streaked onto malt-extract (2% w/v) agar (MEA) plates and allowed to dry overnight. The plates were irradiated for 1.5 min with a UVP Chromato-Vue Transilluminator (302 nm) from a distance of 115 mm. Approximately 80% to 90% of the hyphal fragments were killed by this procedure compared to nonirradiated controls. Surviving colonies were grown on MEA and abnormal colony types selected. Subcultures were also made from mutants that displayed sectoring.

Determination of Colony Morphology

Colony morphologies of all P. placenta isolates were determined after 4 weeks growth on MEA at 27°C. Isolates were described as floccose or appressed.

Extracellular Carbohydrate Production

Cultures were grown in aerated liquid media containing a basal salt solution (Highley, 1973) and variable carbohydrates. Carbohydrates were obtained from the following sources: cellobiose (Sigma, St. Louis, Missouri); xylose, mannose and fructose (Eastman, Rochester, New York); glucose (Fisher, Fair Lawn, New Jersey); galactose (Pfanstiehl Laboratory, Waukegan, Illinois). Two-liter flasks containing 1500 ml media were inoculated with 50 ml hyphal suspension, prepared by blending 2 to 3-week-old cultures of P. placenta grown in 25 ml 1% cellobiose with 100 ml sterile distilled water. Inoculum was standardized in large experiments by mixing serveral suspensions of a given isolate and dispensing the mixture into 50-ml aliquots. Cultures were incubated at 25°C for 10 days unless otherwise specified.

Extracellular carbohydrate was collected from culture filtrates by alcohol precipitation as described by Steiner et al. (1987). The chemical composition of the extracellular carbohydrate isolated from MAD698 was determined by HPLC after acid hydrolysis using the procedure of Pettersen

[2]The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an offical endorsement or approval by the U.S. Department of Agriculture of any product or service to the exclusion of others that may be suitable.

et al. (1984). Protein concentration was determined (Lowry et al., 1951) using bovine serum albumin (BSA) for the standard curve.

The extracellular carbohydrate isolated from MAD698 was digested with a commercial preparation of laminarinase (E.C. 3.2.1.6) (Sigma L9259) derived from Penicillium sp. Ten milligrams of substrate (i.e. the extracellular carbohydrate) were incubated with two units laminarinase in 1 ml 0.1 M McIlvaine buffer, pH 5.0, for 24 hour at 40°C. Laminarin from Laminaria digitata (Sigma L9634) was also used as a substrate. The quantity of reducing sugars released from the substrates, expressed as μmol glucose per hour, was determined by Nelson's modificaiton of the Somogyi method (Nelson, 1944).

Determination of Mycelial Weight, R-glucanase and Laminarinase Activity

Growth curves were developed for P. placenta isolates MAD698 and ME20. The fungi were grown in stationary liquid culture consisting of 1% cellobiose in a basal salt solution (Highley, 1973), inoculated with 8-mm-diam mycelial plugs taken from the margins of 7-day-old colonies. The cultures were incubated at 27°C, and the mycelia collected by vacuum filtration at 3 to 4 day intervals; three replicate cultures were sampled on each harvest date. Mycelial dry weight was determined after drying overnight at 70°C.

The culture filtrates collected by this procedure were analyzed for their ability to degrade laminarin and alkali-resistant glucan (R-glucan). R-glucan was isolated from the cell walls of MAD698 as described by Wessels and Niederpruem (1967). Fungi were grown in aerated 1% cellobiose plus basal salts (Highley, 1973) broth for 10 days at 25°C. The mycelium was harvested by centrifugation (10,000 X g for 20 min) and crushed with a mortar and pestle in liquid nitrogen. The suspension was centrifuged (16,000 X g for 15 min) and the pellet resuspended in 0.1 M acetate buffer, pH 5.0. This process was reported four times with the acetate buffer and three times with deionized water to remove extracellular carbohydrate loosely associated with the cell wall. The supernatant from the final wash was checked for the presence of reducing sugars (Nelson, 1944). Alkali-soluble glucan (S-glucan) was extracted from the washed cell walls with 1 N KOH for 18 hour at 25°C and removed from the R-glucan by centrifugation (12,000 X g for 25 min). The R-glucan, which remained in the pellet, was washed twice with distilled water and freeze dried.

One milliliter of dialyzed culture filtrate was added to 10 mg of substrate (commercial laminarin or R-glucan) suspended in 1 ml 0.1 M McIlvaine buffer, pH 5.0. After 24 hour at 40°C, the mixture was analyzed for the presence of reducing sugars (Nelson, 1944).

Sensitivity to Sterol-Synthesis Inhibitors

The sterol-synthesis inhibitor fenpropimorph (Maag Chemical Company, Vero Beach, Florida) was prepared as a series of stock solutions (v/v in sterile distilled, deionized water) to yield final concentrations of 0.01 to 5.00 μg/ml in molten MEA. Similarly, stock solutions of monensin (Sigma) (w/v in 95% ethanol) were added to molten MEA for final concentrations of 1 to 100 μg/ml. Approximately 20 ml of media were dispensed into 85-mm-diam Petri plates with three replicate plates per concentration per fungal isolate. The sensitivities of the following brown-rot fungi were evaluated: P. placenta--MAD698, L8035spA, ME20, RLG3760R, TRL2556; Gloeophyllum saepiarium--FD18, FP104-D; and Gloeophyllum trabeum--MAD SAVO IS, MAD617. The medium was inoculated with 8-mm-diam agar plugs taken from the margin of 7 to 30-day-old cultures grown on nonamended MEA. Colonies were incubated for 7 days at 28°C. The average colony radius was determined and the percentage of inhibition calculated by comparison to colony radii obtained on nonamended MEA. Dosage-response curves and the ED_{50} value of each isolate were determined by linear regression analysis with a Texas Instruments Advanced Scientific Calculator (TI-60).

RESULTS

Generation of Mutants

Eight mutants were selected (from 410 irradiated colonies) that exhibited abnormal, appresssed colony morphologies when grown on MEA. Three of these, 39-6B, 56-5B, and 51-1B, were subcultured from sectors of colonies that were otherwise floccose. Subcultures were also made from the floccose regions of these three colonies and were designated 39-6A, 56-5A, and 51-1A.

Decay Capacity of P. placenta Isolates

There was no correlation between decay capacity and colony morphology on MEA; all of the mutants retained their ability to decay wood (Table 2). Although the appressed mutants produced no aerial mycelium on MEA, they returned to the more typical floccose morphology when grown on the wood blocks.

Extracellular Carbohydrate Production

All isolates except ME20 produced extracellular carbohydrate, although the quantities produced by replicate cultures of certain isolates could be quite variable. There was no correlation between extracellular

Table 2. Morphology, Decay Ability, and Extracellular Carbohydrate Production by P. placenta Isolates.

Isolate	Colony morphology[a/]	Percent weight loss[b/]	Carbohydrate (mg)[c/] / mycelial weight (g)
RLG3760R	Floccose	65.9	66.0
UV55-5	Appressed	64.8	18.7
MAD698	Floccose	64.3	57.5
UV59-1	Appressed	64.0	11.3
UV39-6A	Floccose	63.6	37.4
UV39-6B	Appressed	63.3	33.2
UV28-18	Appressed	62.6	63.5
UV27-5	Appressed	62.0	15.1
L8035spA	Floccose	58.2	41.6
UV64-4	Appressed	56.6	45.8
UV56-5A	Floccose	55.8	17.6
ME20 X L8035a	Floccose	55.3	57.4
UV32-7	Appressed	54.0	41.4
UV51-1B	Appressed	53.0	76.2
UV51-1A	Floccose	48.5	23.5
ME20	Appressed	5.1	0.0

[a/] Colony morphology on 2% (w/v) malt-extract agar after incubation for 4 weeks at 27°C.

[b/] All readings represent the average of 6 replications. Pooled standard deviation = 7.4 as calculated by Minitab Data Analysis Software, Minitab, Inc., State College, PA.

[c/] All readings represent the average of two replications. Pooled standard deviation = 17.9 as calculated by Minitab Data Analysis Software, Minitab, Inc., State College, PA.

carbohydrate formation and colony morphology on MEA (Table 2); the appressed mutants retained the ability to produce carbohydrate.

The chemical composition of the carbohydrate formed by our standard test isolate, MAD698, was 87.0% glucose, 0.4% mannose, and 12.6% unidentified material. Xylose, galactose, and arabinose were not present. Less than 10 µg protein was detected per miligram of the dried carbohydrate. A commercial preparation of laminarinase released reducing equivalents equal to 12.2 µmol glucose per hour. This same preparation liberated 22.4 µmol glucose per hour from a commerical source of laminarin.

The amount of carbohydrate produced by MAD698 varied with time (Table 3). Little or no carbohydrate was isolated early or late in the growth curve; the largest quantities were formed during the logarithmic phase. Isolate ME20 failed to produce carbohydrate throughout the growth cycle.

The type of carbon source did not greatly influence the amount of extracellular carbohydrate produced by MAD698 (Table 4). The largest quantities of carbohydrate were formed when the fungus was grown on xylose or fructose; growth on galactose, mannose, cellobiose, and glucose resulted in equivalent levels of carbohydrate formation. Little difference was found in carbohydrate production with 0.5% or 1.0% glucose or cellobiose. Isolate ME20 failed to produce extracellular carbohydrate regardless of carbon source or concentration.

Determination of Mycelial Weight, R-glucanase and Laminarinase Activity

Growth curves formed by MAD698 and ME20 in static culture are presented in Figure 1. Both fungi rapidly entered logarithmic growth. Isolate MAD698 continued to increase its dry weight slowly until day 15, after which the cultures became senescent and mycelial weight declined. The dry weight of ME20 decreased rapidly after only 7 days.

The concentrations of laminarinase and R-glucanse detected in the culture filtrates are presented in Figures 2 and 3, respectively. Strain MAD698 produced uniform levels of laminarinase throughout the growth curve. In contrast, ME20 initially formed much lower quantities of this enzyme, but production rapidly increased until day 12 when its activity equaled that of MAD698. At this time, ME20 started to autolyze and mycelial weight declined. Isolate ME20 produced more than twice as much R-glucanase than MAD698 during this period of decline.

Table 3. Dry Weight, Protein and Extracellular Carbohydrate Production in Aerated Liquid Culture by MAD698 and ME20 with Time.[a/]

Day	Dry Weight (g)		Protein (µg/ml)		Carbohydrate (mg/g dry wt)	
	MAD698	ME20	MAD698	ME20	MAD698	ME20
5	0.165[b/]	0.369	20	82	0	0
10	1.050	2.661	20	47	29	0
15	2.108	1.820	33	65	25	0
20	2.688	1.144	20	88	2	0

[a/] Isolates grown in aerated 1% cellobiose plus basal salts at 25°C.

[b/] All readings represent the average of two replications.

Table 4. Effect of Carbon Source on Production of Extracellular Carbohydrate by MAD698.[a/]

Carbon source	Carbohydrate (mg) / Mycelial weight (g)
1.0% galactose	46.1
1.0% xylose	88.3
1.0% mannose	51.3
1.0% fructose	81.9
0.5% cellobiose	49.6
1.0% cellobiose	54.9
0.5% glucose	56.9
1.0% glucose	39.2

[a/] Isolates grown in aerated culture at 27°C for 10 days. Isolate ME20 failed to produce any extracellular carbohydrate regardless of carbon source or concentration.

Table 5. Sensitivities of Brown-rot Fungi to Fenpropimorph and Monensin.[a/]

Species	Isolate	ED_{50} (µg/ml)	
		Fenpropimorph	Monensin
Postia placenta	MAD698	0.28	1.2
	RLG3760R	0.79	1.3
	L8035spA	0.26	1.1
	TRL2556	0.20	1.6
	ME20	0.08	1.7
	ME20 X L8035spA	0.52	1.3
Gloeophyllum saepiarium	FP104-087	0.26	9.5
	FD18	0.25	9.9
Gloeophyllum trabeum	MAD617	0.34	1.5
	MAD SAVO IS	0.31	1.7

[a/] Colonies incubated on fungicide-amended media for 7 day at 28°C. ED_{50} values represent an average of three replications.

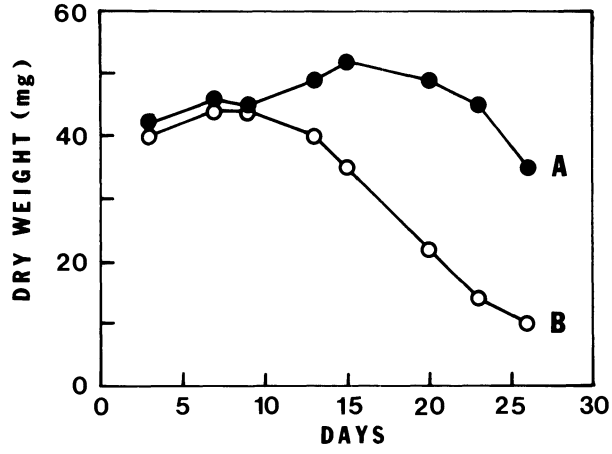

Figure 1. Growth Curve of MAD698 (A) and ME20 (B) Grown in Static Culture of 1% Cellobiose Plus Basal Salts (Highley, 1973). Each Point Represents the Average of Three Replications.

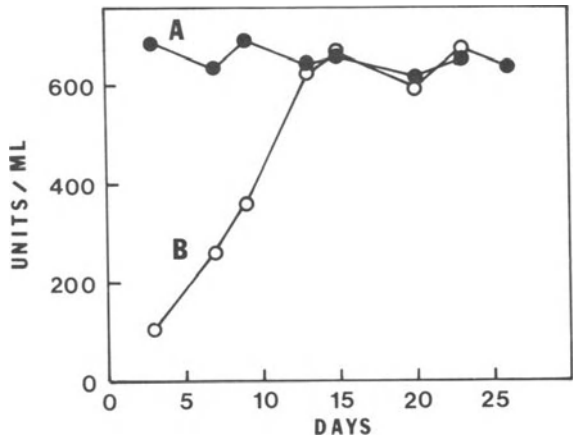

Figure 2. Laminarinase Activity Detected in the Culture Filtrates of MAD698 (A) and ME20 (B) Grown in Static Culture of 1% Cellobiose Plus Basal Salts (Highley, 1973). Each Point Represents the Average of Three Replications.

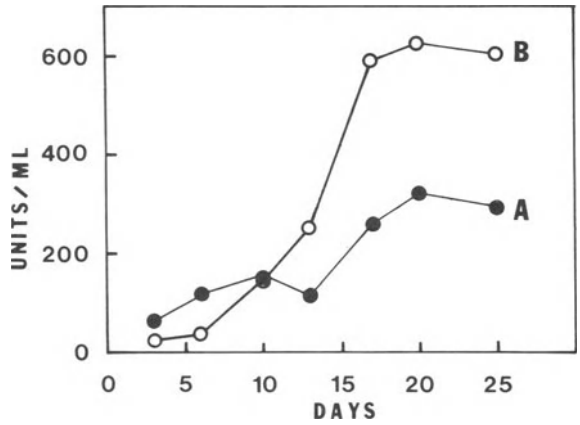

Figure 3. R-glucanase Activity Detected in the Culture Filtrates of MAD698 (A) and ME20 (B) Grown in Static Culture of 1% Cellobiose Plus Basal Salts (Highley, 1973). Each Point Represents the Average of Three Replications.

Sensitivity to Sterol-Synthesis Inhibitors

The sensitivity of different isolates of P. placenta, G. saepiarium, and G. trabeum to the sterol-synthesis inhibitors fenpropimorph and monensin are represented as ED_{50} values in Table 5. Isolates of G. saepiarium were more resistant to monensin that were isolates of P. placenta and G. trabeum, but there was very little intraspecific variation in sensitivities. Such uniformity was not observed among isolates of P. placenta in response to fenpropimorph. Certain isolates (RLG3760R and ME20 X L8035spA) displayed an increased resistance (ED_{50} > 0.5 µg/ml) when compared to the majority of isolates (ED_{50} = 0.20 - 0.28 µg/ml); in contrast, ME20 exhibited an increased susceptibility (ED_{50} = 0.08 µg/ml). Isolates of G. saepiarium responded similarly to the majority of P. placenta isolates (ED_{50} = 0.25 - 0.26 µg/ml), but cultures of G. trabeum were somewhat more resistant (ED_{50} = 0.31 - 0.34 µg/ml).

DISCUSSION

The nondegradative isolate of P. placenta, ME20, failed to produce extracellular polysaccharide in aerated liquid culture regardless of carbon source or concentration. Isolates of P. placenta that degrade wood formed varying amounts of this material. Although the exact chemical nature of this carbohydrate has not been determined, it consisted of 87% glucose. This analysis compares well with the chemical composition of glucan produced by Schizophyllum commune (90%) (Wessels et al., 1972) and Phanerochaete chrysosporium Burds. (92%) (Bes et al., 1987). The carbohydrate was degraded by a commercial preparation of laminarinase, suggesting a β-1,3 linkage of glucose units as described for the glucan of other wood-decay fungi (Axelsson et al., 1968; Bes, et al., 1987; Buchala and Leisola, 1987; Steiner, et al., 1987; Van der Valk, et al., 1977; Kikumoto et al., 1970; Wessels et al., 1972).

The production of glucan varied over the life cycle of MAD698, our standard test isolate of P. placenta, with the largest accumulations occurring during the logarithmic growth phase. Little glucan was harvested from 20-day-old cultures. Such a distribution agrees with that reported from P. chrysosporium and S. commune, where extracellular glucan acts as a storage material that is synthesized when glucose is excessive and metabolized when glucose becomes limiting (Bes et al., 1987; Niederpruem et al., 1977; Wang and Miles, 1964). In white-rot fungi, wood decay is repressed in the presence of simple sugars; excess glucose represses the induction of cellulases, hemicellulases, and the sugar-oxidizing enzymes that produce the hydrogen peroxide necessary for lignin degradation (Bes et al., 1987; Ericksson and Goodell, 1974; Highley, 1987). The removal of

excess glucose by conversion to glucan allows decay to proceed. Although the production of carbohydrate-degrading enzymes by many brown-rot fungi is not repressed by the presence of glucose (Highley, 1987), the formation of extracellular glucan as a storage material would still be an important survival mechanism. The absence of glucan as an extracellular storage material may explain the rapid senescence of ME20 demonstrated in Figure 1.

The relationship between colony morphology, decay ability, and lack of glucan production occurred only for ME20; the eight ultraviolet-induced mutants that superficially resembled ME20 continued to produce glucan in liquid culture and retained the ability to decay wood. The abnormal morphology of these mutants may be associated with nutrition, however, because all of them produced the more typical floccose morphology on wood blocks. The hyphae of ME20 did not vary when grown on different media or on wood.

The fact that ME20 is monokaryotic should not affect its ability to degrade wood. Monokaryons usually retain their degradative capacity and may cause greater weight losses than dikaryotes (Amburgey, 1967, 1969), as demonstrated with monokaryotic isolate L8035spA.

Morphological mutants of other wood-decay fungi have been described that resemble ME20. Amburgey (1967; 1969) reported that certain appressed isolates of Gloeophyllum trabeum did not degrade wood. The most detailed studies have been conducted with the "thin" mutants of Schizophyllum commune. These isolates do not produce aerial mycelia, and the hyphae that are produced are wavy or helical and display unusual branching patterns (Schwalb and Miles, 1967). As with ME20, the "thin" mutants fail to form extracellular glucan in liquid culture, although low levels of polysaccharides are sometimes associated with the hyphal surface (Wessels and Niederpruem, 1967; Wessels et al., 1972; Niederpruem et al., 1977; Schwalb and Miles, 1967). This condition has been associated with a single recessive gene (Raper and Miles, 1958; Leary, 1964), as well as with mutations in the A and B mating factors that relate directly to cell wall synthesis (Wessels and Niederpruem, 1967; Wessels, 1969; Wessels, 1978). 'Thin' mutants are either common A heterokaryons or homokaryons with a primary mutation in the B mating factor. Both conditions result in elevated production of R-glucanase, an enzyme that hydrolyzes the alkali-insoluble glucan of the fungal cell wall (Wessels et al., 1972). The specific activities of lipase and amylase are also elevated in these mutants, although levels of chitinase and proteases are unaffected. The increased activity of these hydrolytic enzymes leads to a depletion of energy storage compounds in the mycelium, including R-glucan, glycogen,

and triglycerides (Wessels, 1978). Such a condition represents a massive loss of energy and resembles the physiology of a normal culture during carbon starvation (Wessels, 1978). A similar situation may exist for ME20. The R-glucanase levels produced by ME20 were much higher than those of MAD698; studies are currently in progress to assess the concentrations of other hydrolytic enzymes. Although similar enzymatic mechanisms may be responsible for the abnormal morphology of ME20 and the S. commune isolates, the genetics of the organisms must be expressed quite differently because P. placenta exhibits unifactorial, rather than bifactorial, sexual incompatibility.

Preliminary observations with scanning electron microscopy have suggested that abnormalities may occur in the hyphal sheath of ME20 (Micales et al., in press). Recent studies of the hyphal sheath of wood-decay fungi (Foisner et al., 1985a, 1985b; Green et al., 1989; Messner et al., 1987) have revealed large quantities of membranous-like structures that may facilitate the decay process (Green et al., 1989). We were therefore interested in determining whether two fungitoxicants that affect membrane properties would elicit differential growth responses in ME20 and other isolates of P. placenta. Fenpropimorph, a morpholine derivative, inhibits the synthesis of ergosterol, the principle sterol of fungal membranes (Weete, 1987). This compound inhibits the activity of two enzymes, sterol Δ^{14} reductase and sterol $\Delta^{8} \rightarrow \Delta^{7}$ isomerase, and leads to the accumulation of abnormal sterols in the membranes. These abnormal sterols alter membrane permeability and function, causing fungal stasis or death (Steel et al., 1989; Weete, 1987). The antibiotic monensin, a polyether carboxylic acid, also alters membrane properties. It serves as an ionophore and forms lipophilic complexes with cations. These complexes are inserted into the fungal membranes, leading to the disruption of endomembrane function, intracellular transport, and sterol biosynthesis (Weete et al., 1989).

Isolates of P. placenta expressed uniform sensitivities to monensin but differential sensitivities to fenpropimorph. Strain ME20 was particularly sensitive to the latter compound. It is unknown whether this sensitivity is due to actual differences in the target enzymes of the biosynthetic pathway, or whether a more indirect effect, such as reduced uptake or increased elimination of the fungitoxicant, is involved (Weete, 1987). One of the proposed roles of the hyphal sheath is the protection of the fungus from adverse environmental or chemical agents (Highley, 1987). Abnormalities in the hyphal sheath may compromise this protection and make the fungus more susceptible to certain chemicals. The uniform response of the isolates to monensin would tend to support a more direct mechanism.

Future studies with additional sterol-synthesis inhibitors should help to determine the specificity of this phenomenon.

SUMMARY

A morphologically atypical strain of the brown-rot fungus Postia placenta, designated ME20, which produces insignificant weight losses in wood, was used to study the mechanisms of decay by brown-rot fungi. Unlike degradative strains, ME20 exhibited an appressed morphology due to an absence of aerial hyphae. The relationship between appressed morphology and decay ability is not necessarily causal, however, because appressed ultraviolet-induced mutants retained the ability to degrade wood. Strain ME20 failed to form extracellular glucan in liquid culture regardless of carbon source or concentration; all other strains of P. placenta, including ultraviolet-induced mutants, produced this material. Cultures of ME20 senesced rapidly compared to a standard test isolate. Strain ME20 also produced elevated levels of alkali-resistant glucanase, a degradative enzyme that hydrolyzes the alkali-insoluble glucan in the fungal cell wall; the incorrect regulation of this enzyme may be indicative of a highly inefficient physiology. Isolates of P. placenta expressed uniform sensitivities to monensin but differential sensitivities to fenpropimorph; both compounds are sterol-synthesis inhibitors. Strain ME20 was particularly sensitive to fenpropimorph. Differences in sterol metabolism and membrane function may explain this heightened sensitivity. This study demonstrates that many diverse physiological pathways may be involved in the development of brown rot.

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Sapstreak Disease and Biodeterioration of Sugar Maple

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INTRODUCTION

Logs and lumber decrease in value due to fungi that stain wood as well as from fungi that cause decay. Sapwood within a living tree is subject to a number of discoloring or staining processes involved with tree defense and fungal infection (Shortle, 1984). Although the use of color to typify wood tissues can result in confusion (Shigo, 1986), changes in color are indicative of distinct metabolic processes that once located may then be more precisely characterized. Sap stains are sapwood tissues altered in color due to fungal activity and may occur in the standing tree, cut lumber, or manufactured product (Scheffer and Lindgren, 1940). The sapstreak disease of sugar maple results in a sap stain in the tree that persists in the wood product. Although wood strength is not affected by sapstreak, the stain lowers the commercial value of affected lumber (Ohman and Spike, 1966).

In addition to the loss of economic value, sapstreak is one of the few diseases that can kill mature sugar maple trees within several years of infection (Hepting, 1944; Roth et al., 1959). Sapstreak disease is caused by the fungus first identified by Hepting (1944) as Ceratocystis virescens (Davidson) C. Moreau (1952) (=Endoconidiophora virescens Davidson (1944)) and considered to be synonymous with Ceratocystis coerulescens (Munch) Bakshi (1950). The disease occurs predominately in sugar maples that are wounded at the root collar or the base of the stem (Houston and Fisher, 1964; Houston and Schneider, 1982; Ohman and Kessler, 1963). Such injuries are associated with road building, forest thinning, and the hauling of sap for maple syrup production.

SYMPTOMATOLOGY

The name sapstreak was applied by Hepting (1944) as descriptive of the disease and has been accepted by subsequent workers due to the characteristic pattern of staining of the wood. The significance of the maple sapstreak staining pattern is seen in comparison to the non-specific discoloration of sapwood induced by wounding (Shigo and Sharon, 1968). Sapwood wounded by drilling and colonized by native inoculum other than the sapstreak fungus produces columns of discoloration that are well-defined and strongly bounded (Fig. 1A). The boundary-setting process that minimizes the volume of functioning sapwood lost as a consequence of wounding and subsequent infection is termed compartmentalization (Shigo, 1984).

A strong compartmentalization response is indicated by well-bounded, evenly edged columns of discoloration of relatively small volume (Shigo, 1984). The least well-defined boundaries are at the top and bottom of each column and appear ragged and attenuated. Lateral, radial column boundaries are sharp and distinct and follow the assemblages of ray cells in the wood. The strongest column boundary, termed the barrier zone, is formed by the vascular cambium following wounding. The barrier zone restricts the development of wound-initiated discoloration to wood present at the time of wounding. Individual trees within a species vary in their innate ability to compartmentalize.

The hallmark of compartmentalization of drill wounds is the production of wound-initiated discoloration that shadows or mirrors the dimensions of the drill bit (Fig. 1A). This strongly bounded discoloration is indicative of an active tree response to injury and infection. In similar wounds infected with the sapstreak fungus, boundaries form but at much greater distances away from the site of wounding (Fig. 1B). Consequently, extensive sapstreak staining can be interpreted as being at least a partial failure of compartmentalization. As with the compartmentalization response itself, inoculated trees vary in the extent of streak development.

In sugar maples, columns of discoloration initiated by drill wounds are medium to dark brown in color. Phenols, in various degrees of oxidation and polymerization, provide the pigments for wound-initiated discoloration. The column of discoloration, when viewed near the wound and in transverse section, generally follows the outline of the drill bit (Fig. 1A). The column boundary layers formed in wood extant at wounding are frequently thin (usually <1 mm) green zones of tissue. Analogous green zones in red maple trees contain elevated levels of phenols, calcium, and potassium salts of organic acid anions (Shevenell and Shortle, 1986).

Field inoculations of trees with the sapstreak fungus provide a tool to discriminate between the non-specific effects of wounding and the specific effects of wound infection by the sapstreak fungus. The sapwood of maple trees infected with the sapstreak fungus and seen in transverse section contains radiating streaks of tan to grey stain with thin radial lines of bright orange and red (Fig. 1B). The outer boundary between a streak and clear sapwood is frequently the same shade of brilliant green as in boundary layers of wound initiated discoloration not associated with sapstreak. The irregular outer or centrifugal boundaries visually suggest the lick of flames. The streaks are brilliant when first exposed, but rapidly oxidize to a muddy brown with little of the detail seen in freshly cut or split sections. With time, the dark, muddy brown fades to a later tan color. In longitudinal section, the stain is a column or series of adjacent columns with ragged, attenuated upper boundaries. The patterns of stain resulting from artifical inoculation are identical to those resulting from natural infections (Hepting, 1944; Houston, 1985). The sapstreak column can extend far into the root system and well up the bole (Houston, 1986; Ohman and Kessler, 1963). Effective barrier zones frequently are formed in response to drill wounds inoculated with the sapstreak fungus, but in those cases in which streaks closely approach the vascular cambium, the cambium is killed and a canker develops.

Freshly exposed streaked maple wood has a lower electrical resistance (R_t) than clear sapwood (Houston and Schneider, 1982). Measurements were recently made of R_t of tissues from five infected trees using the method developed for spruce and fir wood (Smith and Shortle, 1988). The R_t of clear sapwood ranged from 75-90 kiloohms. The R_t of streaked wood ranged from 15-19 kiloohms, indicating an increased concentration of mobile ions. Potassium is the principal element found previously to contribute to changes in wood ionization of maple (Tattar, et al., 1972).

External symptoms of sapstreak infection include stem dieback, the production of fewer, smaller leaves, and the tufting of leaves at the end of branches (Hepting, 1944; Ohman and Kessler, 1963). As the disease progresses, the crown becomes increasingly transparent. Trees vary in their ability to maintain crowns that appear normal while infected with sapstreak; death may occur during the same season as infection, or several years may pass following infection and prior to development of foliar symptoms. Several additional years may be required for tree death following the expression of foliar symptoms.

ECONOMIC IMPACT

The dollar value of sugar maple wood is determined as stumpage, logs,

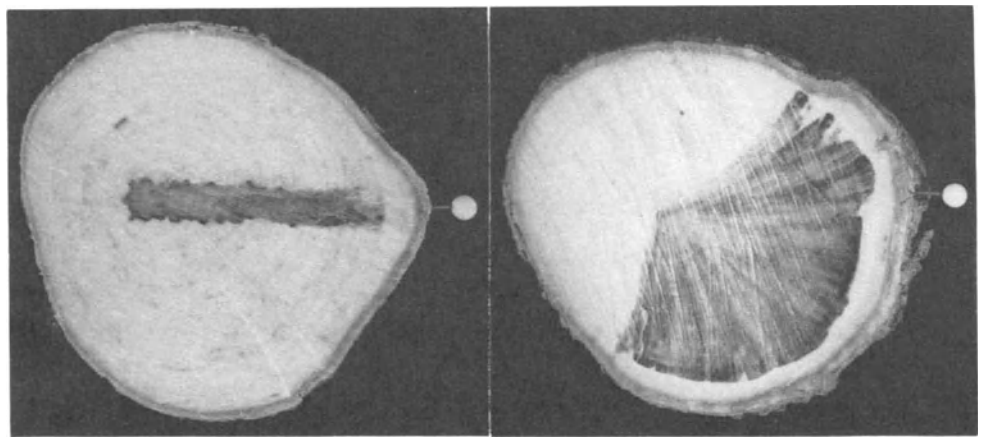

Figure 1. Wound-initiated Discoloration (left) and Sapstreak (right) of Sugar Maple. Both sample disks were obtained 1 cm above a drill wound (marked by a white pin) made during the previous growing season. The wound was either naturally colonized by native, non-sapstreak inoculum (left) or inoculated with a cultured isolate of the sapstreak fungus (right).

and sawn lumber. Stumpage is the dollar value of standing timber. Ohman and Spike (1966) state that salvage harvests of living trees known to be infected with sapstreak is not economical due to the lowering of graded value of logs yielded by those trees. As trees can be infected with sapstreak for several years prior to the development of foliar symptoms, inapproriately high prices for sugar maple stumpage could be set without recognition of the devaluation of the logs and lumber produced.

Within a given set of market conditions, the dollar value of logs or lumber is based on the grade or category of use of each individual log or board. In the grading of sugar maple logs, sapstreak stain is considered a grade defect. Grade defects include those qualities that affect the yield of clear, blemish-free lumber. Grade defects, unlike scalable defects such as rot and checks, are generally not removable during the primary manufacture of lumber from logs (Lockard, et al., 1963). Ohman and Spike (1966) reported that the lowering of the grade classification due to sapstreak reduced the value of logs 35%. Using current prices for sugar maple logs in New Hampshire (Engalichev, 1989), logs with sapstreak stain would lose 40 to 60% of their value.

In determining the grade of sugar maple lumber, a key element is the amount of clear, unstained wood that can be cut from a piece of lumber. For sugar maple lumber that would have been graded as #1 or better in the

absence of stain, sapstreak can reduce the value by more than 70% (Ohman and Spike, 1966; Engalichev, 1989). The value of #2 lumber can be reduced by one-quarter to one-third. In addition to the persistence of the staining pattern, the sapstreak fungus remained viable in certain pieces of air-dried lumber for at least 5 months following the felling of infected trees (Houston, 1986).

The irony is that the tan-colored sapstreak pattern in finished lumber could be considered attractive for certain decorative applications. The sapstreak coloration, when freshly exposed from a living tree, is quite dramatic and could be considered as more desirable than clear, uninfected wood. However, the intense red, orange, and green portions of the stain oxidize within a few minutes and become muddy and unattractive. Unfortunately, there is no method that has been described that would preserve the original colors of the streak and permit the wood to be fashioned into a product.

EPIDEMIOLOGY AND NOMENCLATURE

The frequency of sapstreak disease incidence is not known. Infected maples have been reported in North Carolina (Hepting, 1944; Roth, et al., 1959;), Tennessee (Roth et al., 1959), Michigan and Wisconsin (Kessler and Anderson, 1960; Ohman and Kessler, 1963), Vermont (Houston and Fisher, 1964) and New York (Houston and Schneider, 1982). Individual yellow-poplar trees, one in North Carolina and one in Tennessee, have been reported infected with sapstreak.

A riddle posed by the sapstreak disease is: "why is the disease apparently infrequent in the face of abundant inoculum, plentiful potential infection courts, and numerous susceptible host trees?" A step towards solving the disease incidence riddle may lie in questioning the assumption that the sapstreak fungus is the same as the ubiquitous species reported by Shigo (1962) to rapidly colonize freshly exposed wood surfaces as a saprophyte. A brief examination of the nomenclatural history of the sapstreak fungus introduces the problem and may point to a solution. In his initial description of the disease, Hepting (1944) noted that only one fungus was consistently isolated from the symptomatic streaks. Hepting applied the species name Endoconidiophora virescens, then-recently published by Davidson (1944). Davidson erected the species to acommodate certain isolates obtained from sap stained hardwood logs in North America, and to segregate these isolates from the morphologically similar E. coerulescens that was first described from blue-stained conifer wood in Europe. Davidson (1944) considered that although E. virescens was generally similar to E. coerulescens, the former species was distinct due to physiological differences evident in different volatiles produced by

the two fungi in culture, and to differences of preference for natural substrata. Also, Davidson described a second, smaller type of conidiogenous cell in E. virescens that is absent from C. coerulescens. Hepting (1944) accepted these distinctions and considered that the sapstreak fungus was the same species as the North American sap stain fungus on hardwoods, E. virescens.

Bakshi (1950) properly recognized that for priority and other nomenclatural reasons, the genus Endoconidiophora was a later synonym for the correct genus name, Ceratocystis. Subsequently, Bakshi and other workers formally transferred species from Endoconidiophora to Ceratocystis. Unfortunately, perhaps, Bakshi (1951) rejected Davidson's argument for species segregation and included both the sapstreak fungus and the sap stain fungus of North American hardwoods in the same species as the blue-stain fungus of European and North American conifers. Subsequent monographs on the genus Ceratocystis including the most recent treatment (Upadhyay, 1981) all accept Bakshi's broad concept of C. coerulescens. However, several of the monographs of Ceratocystis, including the one by Upadhyay, express concern about having within the same species the sap stain fungus of North American hardwoods and the blue-stain fungus of conifers. I would extend that concern to question inclusion of the sapstreak fungus with the North American hardwood sap stain fungus (Hepting, 1944).

An element of the answer to the riddle of low disease frequency may be that sapstreak inoculum is not as prevalent as the frequently encountered hardwood sap stain fungus. Cross-inoculation experiments using various isolates of the fungi in question could help determine the validity of including the sapstreak fungus with either or both the sap stain fungus of hardwoods and the blue-stain fungus from conifers. The single published report of inoculations of sugar maple with a hardwood sap stain fungus identified as C. coerulescens indicated that the sap stain isolate did not induce the disease (Roth et al., 1959). Were this finding repeated, it would not automatically exclude conspecifity. The physiological and pathological distinctions could identify prospective isolates for more rigorous morphological investigation. Traditional morphological and taxonomic features could then be compared with physiological differences to determine any consistency of association. The key to unraveling this confusion may lie in the development of a more restricted species concept that accommodates these marked differences in habitat and physiology. Indeed, the use of physiological characters combined with differences in anamorphic (conidial) features is in large part the basis for transferring numerous other species from Ceratocystis

to Ophiostoma (Harrington, 1987). With such precedent set on the generic level, applying analogous distinctions to separations of species within Ceratocystis ought to be acceptable, if warranted.

SUMMARY

The growing interest in the sapstreak disease of sugar maples is based on the loss of value of affected wood and adult tree mortality. The reduction in grade of valuable logs and lumber is a threat to increased utilization of sugar maple wood, especially for the growing export market. Concerns of foreign importers for the potential introduction of new strains of pathogenic fungi need to be addressed. One step in developing an appropriate level of concern rests in clarifying the distinction between the sapstreak fungus and related fungi. This practical need presents an opportunity to test predictions of fungal classification based on physiological and pathological characteristics. Also, this killing disease provides a model system for the study of compartmentalization and its failure in maple.

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Ultrastructural Morphology of the Hyphal Sheath of Wood Decay Fungi Modified by Preparation for Scanning Electron Microscopy

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INTRODUCTION

Our previous attempts to elucidate the structure and extent of the hyphal sheath of wood-decay fungi by scanning electron microscopy (SEM) showed that different preparative methodologies yield differing and frequently conflicting results. Foisner et al. (1985a and b), Highley and Murmanis (1985, 1987), and Green et al. (1989) provided substantial ultrastructural evidence for the presence of extracellular membranous structures that assume a variety of forms, including sheets, tubules, vesicles, and fibers. Evans et al. (1981) reported a fibrillar sheath surrounded by a tripartite pellicle on rapidly growing Bipolaris maydis. Day et al. (1986a and b) also provided evidence for extracellular fungal stuctures, called "fungal fimbriae", which the authors described as primarily proteinaceous. Foisner et al. (1985a and b) analyzed isolated, extracellular membranous structures, which were reportedly composed of carbohydrate, protein, and lipid. These structures were better visualized by transmission electron microscopy (TEM) after treatment with osmium and/or ruthenium red.

Thus, the literature supports the hypothesis that the membranous structures comprising the hyphal sheath form a tripartite lipid bilayer, without phospholipids, associated with protein and carbohydrate. The carbohydrate component is probably fungal glucan in various forms. The

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presence of fungal glucan in a variety of filamentous fungi has been well documented; fungal glucan has been referred to variously as mucilage, extracellular slime, mucoid substance, and mucopolysaccharide. The glucan moiety is composed of 90% glucose residues (Foisner et al., 1985b; Micales and Highley, 1990). Bonfante-Fasolo et al. (1987) suggest that extracellular polysaccharides are rich in low molecular weight sugars, usually lost during conventional procedures for electron microscopy.

The extracellular materials of microorganisms are frequently viewed as contaminants, which should be removed prior to fixation by treatments as simple as washing in an isotonic buffer (Hayat, 1981; Wilcox and Brier, 1987). Often, the presence of adherent slime on the surface of tissues examined by SEM obscures underlying structures (Honegger, 1985; Waterman, 1982).

We consider the term 'hyphal sheath' to refer to a sheath that includes the various structural membranous elements as well as glucan. The hyphal sheath is not confined to only the immediate vicinity of the hyphae, but is ubiquitous and extensively distributed over the lumen surface of the wood cell wall, as is the decay process. Presence of glucans in cell wall and extrahyphal extensions are frequently overlooked (Fleet and Phaff, 1981).

In this SEM study, we utilized several conventional fixation techniques routinely used in TEM for visualizing wood-decay fungi. We conjecture that membrane-specific fixatives enhance preservation of the organized lipid layers in association with protein and glucan. Conventional fixation techniques were supplemented with additives reported to enhance membrane stabilization, e.g. $CaCl_2$, OsO_4, picric acid, saponin, and glutaraldehyde. Cryofixation was also used, as a means of excluding chemical fixation and solvent dehydration.

Central to any ultrastructural study is the acknowledgement of the possible generation of artifacts that are not identical to actual in vivo structures (Crang, 1988). However, the modification of biological structure by a variety of fixation protocols is important from the perspective that certain structures, especially membranes, will respond in a predictable manner, thus revealing their identity. The purpose of this study was to extend our previous observations (Green et al., 1989) of the ultrastructural morphology of the hyphal sheath of brown- and white-rot fungi by SEM using diverse preparative protocols. The results presented here illustrate a variety of morphological modifications of the hyphal sheath observed after fixation and dehydration for SEM. These modifications provide additional evidence for the hypothesis that the hyphal sheath is an extensive extramembranous structure.

MATERIALS AND METHODS

Fungi and Culture Conditions

Wood blocks (8 by 8 by 4 mm) of various species were decayed by different fungi using the ASTM soil-block procedure (ASTM, 1971). Southern yellow pine (*Pinus spp.*) blocks were decayed by *Postia placenta* (Fr.) M.Lars. et Lomb. (isolate # MAD-698), maple (*Acer spp.*) blocks by *Trametes versicolor* (L.:Fr.) Pilat (isolate # MAD-697), and poplar (*Populus spp.*) blocks by *Phanerochaete chrysosporium* Burds. in Burds. et Eslyn (isolate # MAD-461). Wood blocks were removed at selected intervals during the 12-week decay period for examination by SEM.

Fixation

Several fixation schedules were used for decayed wood blocks: (1) cryofixation by rapid quenching in precooled (30 lb/in^2, -21°C) liquid nitrogen (QLN) and lyophilization without chemical fixation; (2) aldehyde fixation--1.0% glutaraldehyde (G) and 4.0% paraformaldehyde (F) in 0.1 M cacodylate buffer (pH 7.2) for 1 to 12 h, double fixation in the same fixative plus 0.05% $CaCl_2$ and 2% OsO_4, the same fixative plus 0.05% $CaCl_2$ and 0.05% saponin (SAP) added (Hayat, 1981), or the same fixative postfixed in 1% OsO_4; (3) chemical fixation in saturated picric acid, 2% paraformaldehyde and 3% glutaraldehyde (PAFG) in phosphate buffer as described by Waterman (1982) for 2 h, or the same fixative postfixed in 2% OsO_4 overnight; and (4) chemical fixation in 1% aqueous OsO_4 vapors for 4 to 8 h in a chemical hood (Cole, 1986).

Dehydration and Drying

Three techniques were used for dehydration and drying: critical point drying, freeze drying, and air drying. For critical point drying (CPD), fixed decayed blocks were dehydrated in a series of increasing concentrations (30, 50, 70, 85, 90, 95, 100%) of ethanol/buffer or acetone/buffer and dried in 5 to 10 changes of liquid CO_2 (transition fluid) in a Balzer's[2] critical point dryer equipped with a high pressure filter (Union Carbide, NJ). For freeze drying (FD), cryofixed decayed blocks were transferred to a precooled cryovessel and lyophilized overnight at -55°C for 12 h.

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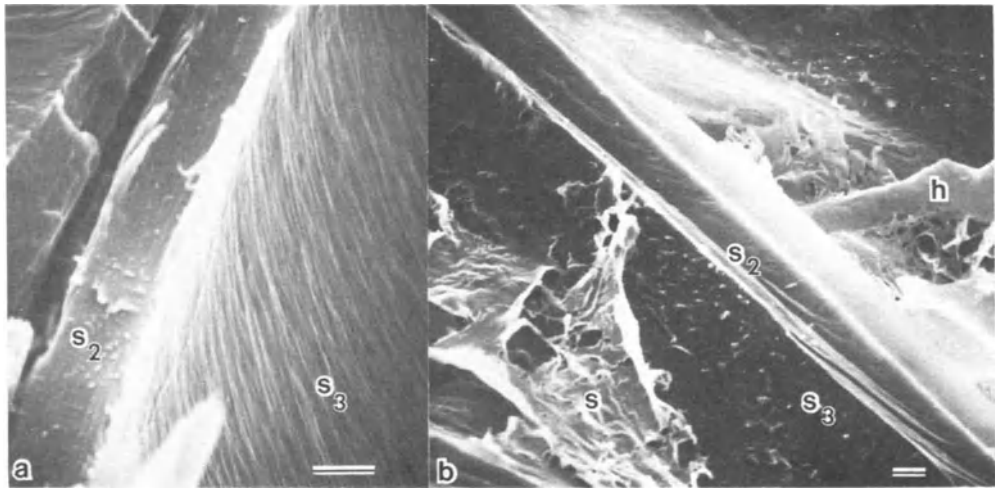

Figure 1. Scanning Electron Micrographs of Undecayed and Decayed Wood of Pinus spp. (QLN-FD). (a) Undecayed Control, (b) Wood Decayed by Postia placenta. Note Prominence of Fiber Angle in S3 Layer (a); (b) Fiber Angle Occluded by Hyphal Sheath (s) Covering S3 Layer and Hypha (h). Scale Bar in (a) = 10 μm; (b) = 5 μm.

Coating and SEM

Dried wood blocks were split longitudinally with a razor blade to expose the radial face and then coated with gold (Au) in a Polaron sputter-coater for approximately 22 s resulting in an Au-layer approximately 65 to 70 A thick. Specimens were examined with a Hitachi S-530 scanning electron microscope at an accelerating voltage of 25 Kv and working distance of approximately 5 to 10 mm.

RESULTS

The ultrastructural features of the hyphal sheath and associated extracellular hyphal structures of the brown-rot fungus P. placenta (Figures 1 to 3, and 4e-f), and the white-rot fungi T. versicolor (Figure 4a-b) and P. chrysosporium (Figure 4c-d) were illustrated by SEM. In general, the hyphal sheath of both brown- and white-rot fungi appeared extensive and similar. Different morphological variations in hyphal sheath structure were observed and were dependent upon the preparative methods used for fixation and dehydration.

The hyphal sheath covered the hyphae and extended out from them onto the lumen surfaces of the wood cell walls. The sheath often appeared disrupted at the hyphal-S3 interface. This was probably due to shrinkage during dehydration (Figure 2b, d, and g). The extensive nature of the

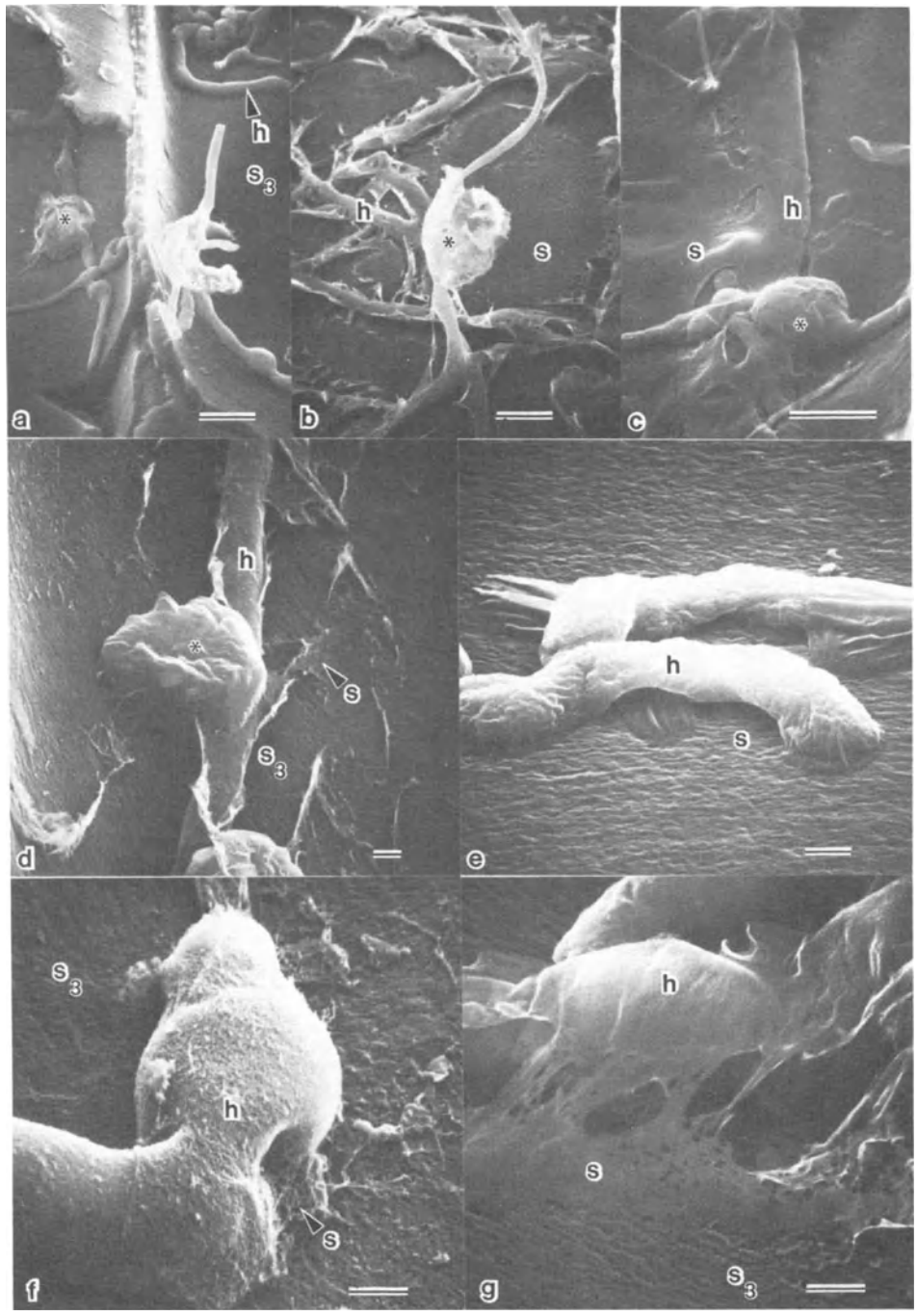

Figure 2. Sequential Modifications of Hyphal Sheath of Postia placenta on Pinus spp. by Various Preparative Methods. (a) GF 1 h-Etoh-CPD, (b) GF 2 h-Acetone-CPD, (c) GF+$CaCl_2$ SAP 12 h-Etoh-CPD, (d) PAFG 2 h-OsO_4 12 h-Etoh-CPD, (e) OsO_4 Vap. 4 h-QLN-FD, (f) GF-Acetone-CPD, (g) GF+$CaCl_2$+SAP 1 h-Etoh-CPD. (*Note Extrahyphal Structures) Scale Bars = 1 μm.

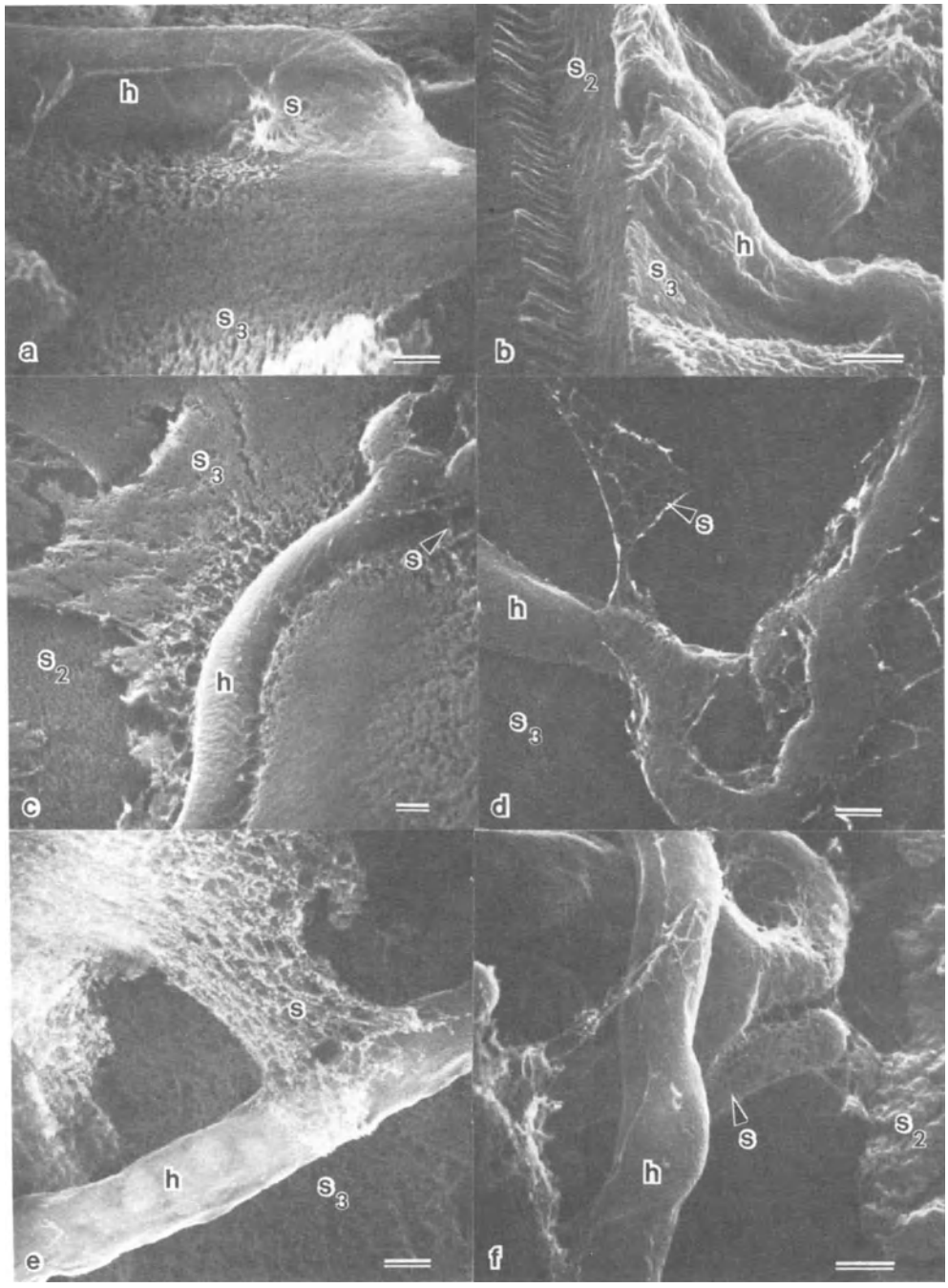

Figure 3. Fibrillar Modifications of Hyphal Sheath of Postia placenta on Pinus spp. by Various Fixation-Dehydration Protocols. (a) Fixed PAFG 12 h-Etoh-CPD, (b) OsO_4 Vap. 4 h-AD, (c) PAFG 2 h-OsO_4 12 h-CPD, (d) GF+$CaCl_2$+OsO_4 8 h-Etoh-CPD, (e) GF-Acetone-CPD, (f) PAFG 2 h-Etoh-CPD. Scale Bars = 1 µm.

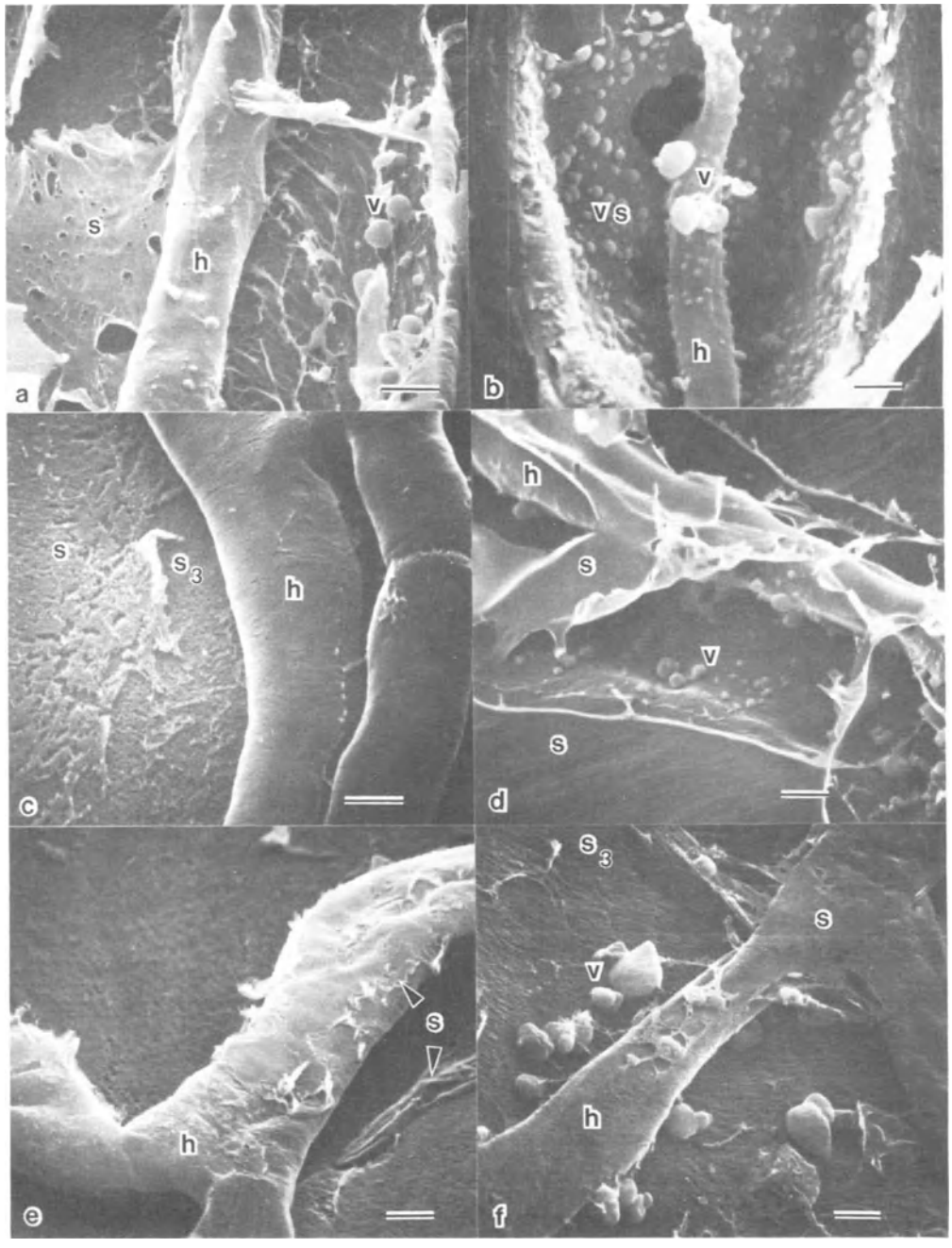

Figure 4. Vesiculated and NonVesiculated Hyphal Sheath of Brown- and White-Rot Fungi. (a-b) Trametes versicolor: QLN-FD; (c-d) Phanerochaetae chrysosporium: (c) GF-Etoh-CPD, (d) QLN-FD; (e-f) Postia placenta: (e) PAFG-Etoh-CPD, (f) QLN-FD. Scale Bars = 1 µm.

hyphal sheath and its ability to cover and occlude the linearity of the fiber angle of the S3 surface layer of the wood cell wall are evident in Figures 1b and 2c, as compared to the sheath of the undecayed control (Figure 1a).

Removal of the hyphal sheath from the outer surfaces of hyphae and the S3 lumen surface of the wood cell wall occurred frequently after conventional chemical fixation and CPD (Figures 2a-f and 3e). Sequential removal of sheath components was commonly observed in different areas of the same wood block, most likely because of partial penetration of fixatives and/or solvents during CPD. Underneath the hyphal sheath, other extrahyphal structures, which appeared convoluted and membranous, were visible (Figure 2a-c,*). Inclusion of "membrane-stabilizing agents" during fixation (that is, picric acid, $CaCl_2$, saponin, OsO_4), as well as increasing fixation times from 2 to 12 h, apparently improved retention of sheath components and structures during dehydration (Figures 2c-e and 3f). Figures 2e and 3b illustrate the apparent collapse of both hyphae and intact smooth sheath covering the S3 layer.

The increased porosity of the S3 layer of the wood cell wall during the decay process was revealed by dehydration in organic solvents and CPD (Figure 3a-f). Extensive fibrillar sheath structure was observed after aldehyde fixation followed by dehydration in either alcohol or acetone and CPD (Figure 3a, c, d, and f). The fibrillar elements of the hyphal sheath extending from the hyphae were similar to the residual wood structures (lignin ?) and associated porosity of the lumen surface of the wood cell wall (Figure 3a, c, and e).

Structural variation or modification artifacts of the hyphal sheath included lamellar sheets (Figures 1b; 2b, d, g; and 4a, d, f), fibrillar arrays extending between the hyphae and the S3 layer (Figure 3d, e, and f), and vesicular structures (Figure 4a, b, d, and f). These modifications were often seen in one preparative method but were absent in others. For example, in both T. versicolor and P. chrysosporium, vesicles were observed after cryofixation (QLN) (Figure 4a, b and d), whereas both lamellar and vesicular structures were absent following CPD (Figure 4c). In P. placenta, however, angular vesiculation was observed only after primary fixation with PAFG, supplemented with $CaCl_2$ and saponin, and followed by CPD (Figure 4f), and never after QLN (Figure 4e) or exposure to OsO_4 vapors. Smooth, extracellular membranous sheets were most consistently observed and were associated with all three wood- decay fungi after QLN, even though these sheets were often disrupted with holes and cracks caused by shrinkage during dehydration (Figures 1b; 2b and g; 3d; 4a, d, and f).

DISCUSSION

This study was a preliminary survey of preparative methodology for fixation and dehydration of decayed wood blocks for examining the hyphal sheath of brown- and white-rot basidiomycetous fungi by SEM. Clearly, wide morphological variation in hyphal sheath structure was observed within and between different preparative methods. This variability was due in part to the biological variability inherent in the interaction of wood-decay fungi and substrate and in part to the partial or sequential modification of hyphal structures during specimen preparation (fixation and dehydration). No single preparative method was adequate for determining sheath structure. All methods produced artifacts. Nevertheless, several general principles emerge from this study with regard to the interpretation of sheath structures.

There are more morphological similarities than dissimilarities in the ultrastructural characteristics of the hyphal sheaths of the three wood-decay fungi. This is in concert with the conclusions of Highley (1987), Foisner et al. (1985), and Green et al. (1989) that the hyphal sheath plays a central and ubiquitous role in wood decay by both the brown- and white-rot fungi, and that it is operative at large distances from the hyphae.

Conventional aldehyde fixation (glutaraldehyde and paraformaldehyde), followed by solvent dehydration and CPD, removed and/or modified the hyphal sheath unless supplemented with membrane-stabilizing agents in the primary fixative or during postfixation. The dehydration steps for CPD included graded ethanol and acetone series to 100% concentration. Unless lipids or carbohydrates are adequately preserved, extensive removal during dehydration can be expected. Acetone is reported to remove 95% of lipids from membranes of mycoplasmas (Razin, 1969).

A universal feature of all cell membranes is the presence of some amphipathic (bimodal) structural molecule, usually phospholipids in animal cells and glycolipid in plant cells (Green and Tzagoloff, 1966). Lipid extraction procedures demonstrate that membrane-associated polar lipids require polar solvents, such as ethanol, to disrupt hydrogen bonds or electrostatic forces between lipid-protein complexes. In addition, alcohol dehydration coextracts sugars, amino acids, and salts (Kates, 1986). Neutral lipids are soluble in acetone, whereas polar lipids are insoluble in cold acetone (Kates, 1986). During lipid extraction, polar and nonpolar lipids are extracted by ethanol and acetone, respectively, and in addition, alcohol disrupts and extracts lipid-protein complexes (Kates, 1986). Foisner et al. (1985a) characterized the hyphal sheath as 90%

glucose residues and the remainder as protein and lipids. Phospholipids were not detected by Foisner et al. (1985a), but this does not exclude a role for structural glycolipids.

Ethanol precipitates extracellular glucan of wood-decay fungi during isolation procedures (Micales and Highley, 1990). Therefore, if lipids were removed by ethanol in CPD, associated glucan would precipitate and/or condense. Little evidence in the literature suggests that the sheath and related structures would undergo similar extraction during QLN, but freezing or freeze-thawing artifacts may be evident. Such modifications, however, are unlikely to represent a large-scale removal of proteins, lipids, or carbohydrates.

Glutaraldehyde is reported to fix biological membranes (Hayat, 1981), but glutaraldehyde or formaldehyde has only a slight reaction with lipids. Glutaraldehyde cross-links proteins without reducing the fluidity of the lipid bilayer; this can result in the formation of vesicles, blisters, and blebs (Crang, 1988). In addition, phospholipids, when present, may be removed and the lipid moiety of membranes destabilized by glutaraldehyde (Hayat, 1981).

In our study, the inclusion of OsO_4 in the primary fixative or during postfixation enhanced preservation of the hyphal sheath. Osmium is essential to prevent lipid loss, but it also hydrolyzes and extracts proteins (Todd, 1986). Osmium increases the visualization of lipids because of osmophillia (TEM) and stabilizes protein cross-links already formed (Cole, 1986). Postfixation with OsO_4 has even been shown to stabilize membranous artifacts produced during conventional fixation with glutaraldehyde (Hayat, 1981). When OsO_4 is used alone as a primary fixative, it may produce more artifacts than it eliminates (Crang, 1988; Sleytr and Robard, 1982). Hyphal sheath structures have also been visualized in TEM with the aid of ruthenium red (Foisner et al., 1985); however, the specificity of this stain is in doubt because it also binds to acidic amino acids and lipids (Todd, 1986). In Streptomyces salivarius, surface fibrils (probably carbohydrates) could not be seen after conventional aldehyde and OsO_4 fixation in TEM. Polymeric acidic polysaccharides and the neutral polysaccharide, dextran, may also be stained by ruthenium red (Handley et al., 1988).

In our study, addition of $CaCl_2$ and SAP to conventional aldehyde fixatives also resulted in better preservation of sheath structures. Divalent cations ($CaCl_2$) at very low concentrations stabilize mycoplasma membranes against osmotic lysis (Razin, 1969) and also minimize lipid loss during dehydration (Hayat, 1981). In addition, cell swelling during dehydration can be minimized by addition of divalent cations to the graded

ethonal/acetone series (Cole, 1986). To some degree, these effects are due to the increased osmolality of the fixative vehicle, eliminating the membranous blebs of hypotonic solutions. Saponin is a mild detergent that solubilizes membranes, but in concert with aldehyde fixation, SAP also stabilizes membrane structure after membrane permeability is altered (Hayat, 1981).

The hyphal sheath appears and behaves like an extracellular membranous structure covered by or embedded in glucan. We observed a variety of artifacts that are inherent in biological membranes, such as cracked and disrupted lamellar sheets and vesicles or blebs. The best SEM representation of the hyphal sheath of P. placenta (MAD-698) is shown in Figure 2c, where a smooth, uninterrupted matrix covers the entire S3 surface of the wood cell lumen. The sheath may be envisioned as a complex fluid structure of high water content.

Correlative TEM evidence in the literature supports the hypothesis of the hyphal sheath as an extracellular membranous structure (Foisner et al., 1985a and b; Highley and Murmanis, 1985; Palmer et al., 1983). Foisner et al. (1985a and b) demonstrated tripartite, extracellular membranous structures using TEM. Recent studies showed that gold-labeled antibodies to ligninase label the extracellular membranes, suggesting that the hyphal sheath plays a role in decay by white-rot fungi (Daniel et. al., 1989; Blanchette et al., 1989).

The removal and modification of the hyphal sheath reveal an extensive fibrillar structure that is likely to represent condensation and precipitation of extracellular fungal protein components of the sheath. Our observations are similar to those of Tsuneda et al. (1987) for Lentinus edodes (Berk.) Sing.; however, these authors identified the structures as cellulose microfibrils. Fibrillar extracellular sheath structures were also observed by TEM in wood-decay fungi (Bracker, 1967; Nilsson et al. 1989), plant pathogenic fungi (Evans et al., 1981), and ericoid mycorrhizal fungi (Bonfante-Fasolo et al., 1987).

Vesicular modifications of the sheath were viewed in all three fungi studied. Vesicles, blebs, and blisters represent some of the most common artifacts visualized in membranes fixed in glutaraldehyde (Hayat, 1981). It is interesting to note that vesicles of the two white-rot fungi studied were seen only after QLN and that vesicles of P. placenta were seen only after CPD. We found little TEM evidence to support vesiculation of the sheath in P. chrysosporium or P. placenta and view these structures as artifacts. However, the numerous regular vesicles of T. versicolor have also been seen in TEM (unpublished results), and we reserve judgement as to

the possible role that these small vesicles play in enzyme delivery and membrane extension in this species.

This paper in no way exhausts the preparative methodology of fixation, dehydration, and preservation of labile biological structures for examination by SEM. Methods designed specifically to fix or remove the glucan component of the hyphal sheath have not yet been developed. Greatly improved preservation of fungal ultrastructure has been obtained by freeze-substitution (Hoch, 1986). Frozen-hydrated specimens better represent the in vivo condition of specimens than specimens prepared by conventional chemical fixation (Read et al., 1982; Beckett et al., 1982). We hope to confirm our observations of the hyphal sheath of wood-decay fungi by these and other methods in the future.

SUMMARY

A preliminary evaluation of different SEM preparative methods was made with specific emphasis on the structural modifications of the extracellular sheath of brown-rot and white-rot wood-decay fungi. The variety of complex sheath structures (sheets, filaments, and vesicles) observed often depended on the preparative method. The hyphal sheath of the three basidiomycetes studied--Postia placenta, Trametes versicolor, and Phanerochaete chrysosporium--was similar and extensive, and it usually covered the entire S3 surface of the wood cell wall. Conventional aldehyde fixation, followed by organic solvent dehydration for CPD, apparently resulted in the largest morphological variation, unless supplemented by membrane-stabilizing agents. Specimens quenched in liquid nitrogen most consistently showed smooth, lamellar hyphal sheath surfaces. These results provide additional evidence for a "structural membrane element" of the hyphal sheath of wood-decay fungi, which can be sequentially removed and modified by SEM preparative methodology. A more precise understanding of the interaction of fixatives, stains, and solvents with the hyphal sheath components will be required to better approximate the in vivo morphology of the hyphal sheath.

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Biological Control of Wood-Attacking Fungi Using Bacteria

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INTRODUCTION

Soon after felling, wood is susceptible to colonization by wood-attacking fungi. Infection often occurs within a few days after cutting and may continue to progress in the wood, even though the wood is processed into preservative-treated products, such as poles, railroad ties, and construction timbers. The protection of unseasoned timbers against wood-attacking fungi should not be neglected, because the quality and durability of wood products depend on good care of the unseasoned timbers.

Pentachlorophenol formulations have traditionally been used to protect unseasoned timbers but environmental concerns have severely curbed the use of this material. Furthermore, the use of chemical preservatives for such a short period as unseasoned timber requires (usually 4 to 6 months) is expensive. These objections may be overcome by using biological control agents, which are now being seriously considered for wood protection.

Results have been sufficiently promising in previous studies using bacteria to control sapwood inhabiting fungi to warrant further study (Benko, 1988, 1989; Bernier et al., 1986). In this study, we report on the effectiveness of bacteria as biological control agents against blue stain and mold fungi as well as brown- and white-rot decay fungi.

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[1]The Forest Products Laboratory is maintained in cooperation with the University of Wisconsin. This article was written and prepared by U.S. Government emplpoyees on offical time, and it is therefore in the public domain and not subject to copyright.

This study was done in cooperation with Oregon State University.

MATERIAL AND METHODS

Test Fungi

Four representative wood-attacking fungi were selected as test fungi: Postia placenta (Fries) M. Larsen et Lombard, a brown rot; Coriolus versicolor (L.ex Fr.), a white rot; Ceratocystis coerulescens (Munch) Bakshi, a blue stain; and Trichoderma harzianum Rifai, a mold.

Screening Tests

Before testing on wooden samples, we screened almost 100 known bacteria, which produce metabolites suspected of being fungitoxic, and more than 100 unknown bacteria (Buchanan and Gibbons, 1774; Reiner, 1982). The antagonistic activity of bacteria towards wood-attacking fungi was determined on yeast malt agar (yeast extract 4.0 g, malt extract 10.0 g, dextrose 4.0 g, agar 20.0 g, distilled H_2O 1000 ml).

For treatment of wood blocks, we selected six bacteria as the best in controlling one or more type of wood-attacking fungus. The bacteria were Pseudomonas cepacia (Burkholder) Palleroni and Holmes, Streptomyces rimosus Sobin et al., St. rimosus forma paromomycinus Sobin et al., St. chrestomyceticus Canevazzi and Scotti, Streptoverticillium cinnamoneum forma azacoluta (Benedict et al.) Baldacci, and Xenorhabdus luminescens Thomas and Poinar.

Treatment of Wood Blocks

Sporulation agar (broth) (yeast extract 1.0 g, beef extract 1.0 g, tryptose 2.0 g, $FeSO_4$ trace, glucose 10.0 g, distilled H_2O 1000 ml), pH 7.2, was used to grow bacteria for antibiotic production. The six bacteria were separately grown on a shaker at 150 rpm (New Brunswick Scientific Co.) at room temperature for 7 days. The bacterial cultures were then centrifuged for 30 min at 1,600g, and the supernatants mixed together to produce a bacterial solution, which was used for dipping wooden samples, Pinus spp. (southern yellow pine), for 1 hour.

Decay and Stain Tests

The treated blocks were placed on glass rods on top of mycelial mats of wood-attacking fungi, which covered malt agar (30 g malt, 15 g agar, 1000 ml distilled H_2O) in petri dishes.

The test and control samples were incubated in a dark cultivation chamber at 27°C and 70% relative humidity for 8 weeks.

Weight loss in blocks was measured for Postia placenta and Coriolus versicolor, and degree of staining was recorded for Ceratocystis coerulescens and Trichoderma harzianum.

The degree of staining was rated as follows:

0 = Unstained wood, no visible sign of staining on the surface.

1 = Slightly stained wood, individual tiny blue stained spots with a maximum diameter of 2 mm.

2 = Moderately stained wood, at least one-third of the surface stained or staining in lines up to one-half of the whole surface.

3 = Heavily stained wood, more than one-half of the surface stained.

RESULTS

The average weight loss of samples treated with the bacterial solution, exposed for 8 weeks to Postia placenta, was only 0.11%, while the average weight loss of the control samples was 61.44% (Table 1).

Excellent results were also obtained with Coriolus versicolor, where the average weight loss of treated samples was 0.25% and 11.82% for the control samples.

Although some samples tested with the bacterial solution exposed to Ceratocystis coerulescens showed individual tiny blue stained spots, the average degree of staining was still 0. The control samples were heavily stained with an average degree of staining of 3 (Table 2).

Two of the treated samples exposed to Trichoderma harzianum showed no sign of the discoloration, but others showed slight staining of wood. The average was 0 to 1, which means that occassionally tiny green stained spots occurred on the pine blocks, but not larger than 2 mm. The control samples were heavily stained over the entire surface of the samples, with an average degree of staining of 3.

DISCUSSION

This work demonstrates that bacteria have promise as biological control agents in wood preservation. After testing a few hundred different bacteria, we conclude that it will be very unlikely to find a single bacterium that will be effective against all the various types of wood-attacking fungi. The results of our work show that to control a wider spectrum of attacking fungi, it will be necessary to use combinations of different bacteria. Furthermore, for success in the field, it would be beneficial to obtain a better understanding of biological control mechanisms so as to maximize the establishment and controlling activities of artifically applied delivery systems.

Table 1. Weight Loss Produced in Pine Blocks Treated With a Bacterial Solution After Eight Weeks of Exposure to *Postia placenta* and *Coriolus versicolor*.[a]

Treatment	Fungus	Block number	Weight loss range (%)	Average weight loss (%)
Bacterial solution	*Postia placenta*	1 2 3 4 5	0.00-0.16	0.11
Control	*Postia placenta*	6 7 8 9 10	56.97-65.32	61.44
Bacterial solution	*Coriolus versicolor*	11 12 13 14 15	0.00-0.54	0.25
Control	*Coriolus versicolor*	16 17 18 19 20	9.81-12.92	11.82

[a]Test samples, *Pinus spp.*, treated with bacteria solution consisting of *Pseudomonas cepacia*, *Streptomyces chrestomyceticus*, *St. rimosus*, *St. rimosus* forma *paromomycinus*, *Streptoverticillium cinnamoneum* forma *azacoluta*, and *Xenorhabdus luminescens*.

Table 2. Discoloration of Pine Blocks Treated With Bacteria Solution After Eight Weeks of Exposure to *Ceratocystis coerulescens* and *Trichoderma harzianum*.[a]

Treatment	Fungus	Block number	Degree of staining[b]	Average degree of staining
Bacterial solution	*Ceratocystis coerulescens*	1	0	0
		2	0-1	
		3	1	
		4	0	
		5	0	
Control	*Ceratocystis coerulescens*	6	3	3
		7	3	
		8	3	
		9	3	
		10	3	
Bacterial solution	*Trichoderma harzianum*	11	1	0-1
		12	0	
		13	0	
		14	1	
		15	0-1	
Control	*Trichoderma harzianum*	16	3	3
		17	3	
		18	3	
		19	3	

[a] Test samples, *Pinus spp.*, treated with bacteria solution consisting of *Pseudomonas cepacia*, *Streptomyces chrestomyceticus*, *St. rimosus*, *St. rimosus* forma *paromomycinus*, *Streptoverticillium cinnamoneum* forma *azacoluta*, and *Xenorhabdus luminescens*.

[b] 0 = Unstained wood
1 = Slightly stained wood
2 = Moderately stained wood
3 = Heavily stained wood

SUMMARY

This paper discusses the possibilities of using bacteria for biological control of wood-attacking fungi. The following wood-attacking fungi were used: Postia placenta, a brown rot; Coriolus versicolor, a white rot; Ceratocystis coerulescens, a blue stain; and Trichoderma harzianum, a mold. A mixture of six bacteria from the genera Pseudomonas, Streptomyces, Streptoverticillium, and Xenorhabdus was strongly antagonistic against the wood-attacking fungi. Southern yellow pine (Pinus spp.) blocks treated with bacterial solution suffered less than 1% weight loss after 2 months exposure to the brown- or white-rot fungus and were almost unattacked by the blue stain or mold fungus in the laboratory tests.

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Ionization of Wood During Previsual Stages of Wood Decay

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INTRODUCTION

The wood decay process has both visual and previsual stages. Visual stages include both wood discoloration and the loss of structural integrity. Prior to the appearance of visual stages, wood undergoing the decay process is altered. These previsual alterations affect wood properties such as the accumulation of ions and decay resistance in vitro (Shortle and Smith, 1987; Smith, 1987).

Shigometry is a method that can detect the decay of wood in trees and wood products during the previsual stages of decay as well as the visual, advanced stages (Shigo and Shortle, 1985; Shortle and Smith, 1987; Shortle and Hill, 1987). The accumulation of ions in wood (wood ionization) during infection by whiterot and brownrot fungi was studied in Douglas-fir wood in vitro and found to occur long before the development of visual decay symptoms (Shortle, 1982). The purpose of this study was to determine changes taking place during the early and late previsual stages of decay in Douglas-fir, and to compare the results in Douglas-fir to four other species--red cedar, red pine, black cherry, and red oak.

MATERIALS AND METHODS

Early previsual stages of decay of sapwood and heartwood of Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) at 3 and 10 days of incubation were produced in agar-block chambers using 10 x 20 x 20 mm (20 X 20 mm end grain) blocks as previously described for the late previsual stage, 56 days, and the early visual stage, 112 days, of decay (Shortle, 1982). Early previsual decay at 14 days in heartwood of four other tree species, red cedar (Juniperus virginiana L.), red pine (Pinus resinosa Aiton), black cherry (Prunus serotina Ehrh.), and red oak (Quercus rubra

L.), was also produced in agar-block chambers using 50 x 10 x 5 mm (10 x 5 mm end grain) blocks placed tangential face down as previously described for spruce and fir (Shortle and Smith, 1987). The whiterot fungus, Coriolus versicolor (L. ex Fr.) Quel., and the brownrot fungus, Postia placenta (Fr.) Larsen & Lombard, were used as inoculum.

When Douglas-fir blocks were harvested and analyzed, five replicate blocks were used to determine both the water content as percent dry weight and percent weight loss due to decay (Shortle, 1982). Another five replicate blocks were used to determine tissue electrical resistance as previously described (Shortle, 1982). The oven-dried blocks were ground in a Wiley mill to pass a 850-um sieve.

The ground tissue was used to determine extract electrical resistance, pH, total soluble phenol concentration, and mild (1% NaOH) alkali solubility. Extract electrical resistance of the early previsual stage of decay in Douglas-fir sapwood and heartwood at 3 and 10 days was determined using 0.2 g dry wood/5 ml deionized, distilled water heated to 100C for 1 hr with stirring at 20 and 40 min. Alternatively, extract electrical resistance of the late previsual (56 day) and early visual (112 day) stages of decay in Douglas-fir had been previously determined using 2 g dry wood/100 ml distilled deionized water heated to 121C for 20 min (Shortle, 1982). Extract solutions made by either method were cooled to 25 $\pm$ 2C and measurements of electrical resistance were made using a twisted wire electrode calibrated with a 1.0 mM KCl solution. The twisted wire electrode was the same electrode used to determine the electrical resistance of the freshly harvested wood blocks in which a 2.4 mm hole was drilled from the tangential face through the block center (Shortle, 1982). Measurements taken with the twisted wire electrode were done with a Shigometer Model 7950.

In order to obtain consistent readings with the twisted wire electrode in solution, it was necessary to precondition the electrode by taking 20-30 measurements in the 1.0 mM KCl standard solution, alternating with distilled, deionized water. During this preconditioning phase, readings would decrease at first, then become stable for the next 30 to 60 measurements, after which a further decrease in readings may occur. During the stable phase, the KCl standard yielded readings of 35 k $\pm$ 2% ($n = 15$, $p < 0.05$). Measurements of extract electrical resistance were taken during the stable phase alternatively rinsing with distilled, deionized water and taking a standard reading in KCl solution after every four measurements.

The measurements of extract electrical resistance was markedly improved by using stainless steel double pin electrodes attached to a

digitized Shigometer Model OZ-67 (Shortle and Smith, 1987). Readings proved to be quite stable and this method was used to measure the extract electrical resistance of species other than Douglas-fir. Extracts of these species were prepared using 0.3 g dry wood/20 ml distilled, deionized water heated to 90C for 1 hr with stirring at 15 and 30 min. After gravity filtration, the cooled solution was measured using 10 ml of extract in a 50 ml beaker as previously described (Shortle and Smith, 1987).

Measurement of pH was made for Douglas-fir using 1.0 g dry wood/6 ml distilled, deionized water (Stamm, 1961). Those for other species were made on the same extract used to measure extract electrical resistance.

Total soluble phenol concentration was determined on 76% aqueous ethanol extracts made using 20 mg dry wood/4 ml 76% ethanol at 70C (heater block) for 1 hr with gentle vortexing at 15 and 30 min. After gravity filtration and cooling, 0.25 ml samples of extract were used in the Folin-Ciocalteau method (Horowitz, 1960) except that only one-half the standard amount of reagent was used.

Mild alkali (1% NaOH) solubility was determined using a modification of ASTM D1109-84 (American Society for Testing and Materials, 1987). Duplicate 0.2 g dry ground wood samples were weighed and placed in large (25 mm diameter x 150 mm long) test tubes to which 15 ml 1% (w/v) NaOH was added. A stirring rod was added to the tubes and they were placed in a boiling water bath for 1 hr with stirring at 10, 15, and 25 min. After 1 hr, the extracted wood was recovered in tared, fitted glass crucibles, porosity M, under suction filtration. The extracted wood was rinsed with two 10-ml portions of boiling, distilled, deionized water, two 10-ml portions of 10% (v/v) acetic acid, and two 10-ml portions of distilled, deionized water. Crucibles were then oven-dried at 102C overnight and the percent solubility was determined as (initial oven-dried weight minus final oven-dried weight)/initial oven-dried weight x 100.

Samples of Douglas-fir wood were taken from a one meter section of a utility pole in which visible brownrot was observed when the section was ripped in half longitudinally. A chisel was used to remove 50 x 10 x 5 mm (10 x 5 mm cross grain) sections from the brownrotted inner heartwood and at distances of 6, 12, and 17 cm along the grain in advance of visibility decayed wood. A parallel set of 4 samples was chiselled from outer heartwood which looked sound, and another parallel set of 4 samples from preserved sapwood. The 12 samples were ground in a Wiley mill and used to determine extract electrical resistance and 1% NaOH solubility as already described for Douglas-fir blocks taken from agar-block chambers.

Means of observations within each stage of decay development were

compared to non-inoculated controls by analysis of variance. Statistical differences are reported along with the appropriate level of probability for each experiment.

RESULTS

Decay of Douglas-fir blocks in the agar-block chambers caused a weight loss of 15% or more after 112 day incubation with the decay fungi (Table 1). These blocks were visibly decayed. Many of the blocks crumbled while being drilled to determine tissue electrical resistance. Other blocks incubated from 3 to 56 days did not show obvious visual indications of decay and had mean weight losses of 10% or less.

Table 1. Weight Loss of Douglas-fir Sapwood and Heartwood Due to Decay by a Whiterot Fungus, Coriolus versicolor, and a Brownrot Fungus Postia placenta.

Tissue	Inoculum	Mean weight loss, % dry wt.[a] Incubation period, day			
		3	10	56	112
Sapwood	Control	0	0	0	0
	C. versicolor	0	1	10	22
	P. placenta	0	0	8	18
Heartwood	Control	0	0	0	0
	C. versicolor	0	0	6	16
	P. placenta	0	0	9	15

[a]Mean of 5 observations, except sapwood control (4 obs at 56 days, 3 obs at 112 days).

Extract electrical resistance indicated that a significant increase in soluble ion content (significant decrease in electrical resistance) was caused by the brownrot fungus, Postia placenta, on sapwood and heartwood after 3 days and by the whiterot fungus, Coriolus versicolor, on sapwood after 3 days and on heartwood after 10 days (Table 2). These differences in ion content between infected tissue and sound controls continued thereafter throughout the previsual (3-56 days) and into the visual (112 days) stages of wood decay.

Table 2. Electrical Resistance of Water Extracts of Douglas-fir Sapwood and Heartwood in Progressive Stages of Decay.

Tissue	Inoculum	Mean extract electrical resistance, k [a] Incubation period, day			
		3	10	56	112
Sapwood	Control	62	59	80	80
	C. versicolor	48*	33*	41*	33*
	P. placenta	30*	31*	31*	27*
Heartwood	Control	72	81	90	90
	C. versicolor	82	56*	40*	43*
	P. placenta	46*	31*	30*	27*

[a]Mean of duplicate observations at 3 and 10 days and at 56 and 112 days were determined by different methods of hot water extraction and two different wood : water ratios, 1:25 and 1:50 respectively. Extracts were cooled to room temperature (25 ± 2C). Measurements were made using a twisted wire electrode standardized with a 1.0 mM KCl solution reading 35 ± 1 k (95% confidence limits, n = 15). Asterisk (*) indicates that a mean differs significantly from its control within a given incubation period by analysis of variance ($P < 0.01$).

Detection of the increased ion content in the solid wood blocks lagged behind that of the extracts, except in the case of the brownrot in sapwood treatment in which measurements of electrical resistance were uniformly on-scale and low at 3 days (Table 3). At 10 days whiterot in sapwood and brownrot in heartwood were readily detected by decreased tissue electrical resistance. As in the extracts, whiterot in heartwood was the last to come on-scale at 14 days in a previous experiment (Shortle, 1982). All treatments were readily detectable by differences in tissue electrical resistance in the late previsual (56 days) and visual (112 days) stages of decay.

Table 3. Electrical Resistance of Blocks of Douglas-fir Sapwood and Heartwood in Progressive Stages of Decay.

Tissue	Inoculum	Mean tissue electrical resistance, k [a] Incubation period, day			
		3	10	56	112
Sapwood	Control	+/380	198 ± 95	240	190
	C. versicolor	+/137	26 ± 5	52 ± 44	26
	P. placenta	22 ± 13	45 ± 24	72 ± 66	33
Heartwood	Control	+	+	+	+/473
	C. versicolor	+	+/315	63 ± 33	29
	P. placenta	+/217	65 ± 24	80 ± 18	34

[a] Five observations off-scale (>500 k) are indicated at +; some off-scale and some on-scale observations are indicated as +/mean electrical resistance. Mean of 5 on-scale observations are given with 95% confidence limits (sapwood controls had fewer than 5 observations due to contaminated blocks, 1 at 56 days, 2 at 112 days). Of 20 decaying blocks at 112 days, 13 fell apart upon drilling so that only 7 observations were made overall averaging 31 ± 4 k .

When the water content of solid wood was 65% or greater, all tissue electrical resistance measurements taken with the twisted wire electrode were on-scale and differences between infected wood and sound controls were evident (Table 4). Blocks with mean water content of 55% or less gave off-scale or highly erratic values of electrical resistance. The water content of whiterot in heartwood was 69% at 14 days when measurements were uniformly on-scale (Shortle, 1982). Thus, we see that the accumulation of ions determined in water extracts (Table 2) precedes the accumulation of free water (Table 4) needed for good electrode contact necessary to obtain useful electrical detection of wood infection (Table 3).

Table 4. Water Content of Blocks of Douglas-fir Sapwood and Heartwood in Progressive Stages of Decay.

Tissue	Inoculum	Mean water content, % dry wt.[a] Incubation period, day			
		3	10	56	112
Sapwood	Control	55 ± 12	65 ± 14	74	75
	C. versicolor	44 ± 5	99 ± 16	134 ± 11	173 ± 6
	P. placenta	104 ± 12	126 ± 12	141 ± 9	171 ± 17
Heartwood	Control	41 ± 6	41 ± 7	40 ± 5	42 ± 9
	C. versicolor	52 ± 38	38 ± 8	135 ± 18	169 ± 17
	P. placenta	44 ± 2	121 ± 6	136 ± 24	163 ± 19

[a] Mean of 5 observations and 95% confidence limits for water content expressed as percent dry weight. Sapwood controls had fewer than 5 observations due to contaminated blocks, 1 at 56 days, 2 at 112 days.

Ions accumulating in wood infected by the brownrot fungus are associated with markedly increased acidity (drop in pH) at all stages of decay (Table 5). Whiterot infection is associated with mildly acidic conditions in late previsual (56 days) and visual (112 days) samples.

Table 5. Acidity Measured as pH of Water Extracts of Douglas-fir Sapwood and Heartwood in Progressive Stages of Decay.

Tissue	Inoculum	pH[a] Incubation Period, day 3	10	56	112
Sapwood	Control	5.2	4.5	5.4	5.3
	C. versicolor	5.3	4.3	4.3	4.4
	P. placenta	3.4	3.0	3.8	3.7
Heartwood	Control	4.5	3.7	5.0	5.3
	C. versicolor	4.0	4.4	4.4	4.5
	P. placenta	3.8	2.7	3.5	3.7

[a]Duplicate observations using 1 g ground wood/6 mL distilled, deionized water.

During early stages of infection, a significant decrease in soluble phenol concentration occurs with both brownrot and whiterot (3 days in heartwood, 10 days in sapwood) (Table 6). The trend of decreased phenol content continues during advancing whiterot infections, but reverses to a significant increase in soluble phenol concentration during brownrot.

A marked and significant increase in mild alkali solubility, indicating a drop in average degree of polymerization, was observed by the late previsual (56 days) stage of brownrot infection (Table 7). A small, but significant, increase of mild alkali solubility was observed for whiterot infection on sapwood only.

Determination of extract electrical resistance, extract pH, and soluble phenol concentration during the early previsual (14 day) stage of whiterot and brownrot infection on the heartwood of four tree species supported the same trends observed in Douglas-fir (Table 8). Brownrot infection resulted in decreasing the electrical resistance faster than whiterot infection in heartwood and the decrease was associated with increased acidity. A 14-day incubation period was not long enough to see a marked change in soluble phenol concentration except for a significant

Table 6. Concentration of Soluble Phenols in Aqueous Ethanol Extracts of Douglas-fir Sapwood and Heartwood in Progressive Stages of Decay.

Tissue	Inoculum	Mean soluble phenol concentration, mg/g[a] Incubation period, day			
		3	10	56	112
Sapwood	Control	8.2	9.4	9.1	-
	C. versicolor	8.6	7.4*	7.0*	5.9
	P. placenta	8.3	7.9*	12.7*	15.9
Heartwood	Control	27.7	23.7	24.8	22.4
	C. versicolor	21.9*	18.7*	-	9.2*
	P. placenta	22.6*	26.3*	30.1*	-

[a]Mean of triplicate observations of total phenols soluble in 76% ethanol expressed as mg gallic acid standard/g oven-dry wood. Asterisk (*) indicates that a mean differs significantly from its control within a given incubation period by analysis of variance ($P < 0.01$).

Table 7. Mild Alkali Solubility of Douglas-fir Sapwood and Heartwood in Progressive Stages of Decay

Tissue	Inoculum	Mean 1% NaOH solubility, % dry wt[a] Incubation period, day			
		3	10	56	112
Sapwood	Control	13	13	15	-
	C. versicolor	13	14	18*	18
	P. placenta	15	17	26*	32
Heartwood	Control	15	15	15	15
	C. versicolor	15	15	-	16
	P. placenta	16	19	29*	-

[a]Mean of duplicate observations of dilute alkali solubility expressed as percent dry weight. Asterisk (*) indicates that a mean differs significantly from its control within a given incubation period by analysis of variance ($P < 0.01$).

Table 8. Comparison of Extract Electrical Resistance (R_e), pH, and Soluble Phenols (ArOH) of Heartwood of Four Tree Species After 14 Day Incubation with Decay Fungi.

Tissue	Inoculum	Mean at 14 days		
		R_e	pH	ArOH
		k		mg/g
Red cedar	Control	30	4.8	18
	C. versicolor	24*	5.2*	18
	P. placenta	16*	4.0*	16
Red pine	Control	27	5.1	3
	C. versicolor	21*	5.3	3
	P. placenta	9*	3.7*	7*
Black cherry	Control	42	5.0	28
	C. versicolor	35*	5.2*	22
	P. placenta	20*	4.3*	24
Red oak	Control	26	4.3	14
	C. versicolor	17*	4.4	9*
	P. placenta	15*	3.9	11

[a]Mean of duplicate observations taken on extracts of ground wood after 14 days incubation. Asterisk (*) indicates a mean differs significantly from its control ($P < 0.01$). R_e = extract electrical resistance using 0.3 g wood/20 mL H_2O; pH taken on same extract; ArOH = soluble phenols in 76% ethanol extract using 20 mg wood/5 mL solvent.

increase in phenol concentration in red pine heartwood caused by the brownrot fungus, P. placenta, and a significant decrease in phenol concentration in red oak caused by the whiterot fungus C. versicolor.

Investigation of a Douglas-fir utility pole section with visual stages of brownrot in the heartwood produced results consistent with the in vitro experiments (Table 9). Samples taken from the pole indicates that the extract electrical resistance of (1) inner heartwood with visible brownrot, (2) inner heartwood which appeared sound and was located 6 cm along the grain from visible brownrot, and (3) outer heartwood, which looked sound, across the grain from visible brownrot. The mild alkali solubility of heartwood with visible brownrot was 3X greater than preserved sapwood. The mild alkali solubility of the sample 6 cm away along the grain was 2X greater than preserved sapwood, as in the case of the late previsual stage of brownrot in Douglas-fir (Table 7). The mild alkali solubility of outer heartwood across the grain was only slightly greater than preserved sapwood, as in the case of the early previsual stage of brownrot on Douglas-fir (Table 7). Both samples of wood that looked sound appeared to be in the previsual stages of brownrot with decay advancing more quickly along the grain of the wood than across the grain.

DISCUSSION

Determination of extract electrical resistance indicated a rapid accumulation of readily soluble ions during the early previsual stages of brownrot and whiterot (Table 2). Changes in the electrical resistance of tissues (Table 3) lagged behind those of extracts (Table 2) due to a lag in wetting (Table 4). It is probable that the accumulation of soluble organic anions, or their potassium salts, brought about by the activity of the brownrot and whiterot fungi contribute to the wetting of the wood blocks during the early previsual stages of decay. Brownrot fungi known to produce simple organic acids appeared to be more efficient than whiterot at wetting and swelling dried wood, especially the difficult to wet heartwood of Douglas-fir. This is consistent with the fact that brownrot fungi are more prevalent in situations where wood is dry, and whiterot fungi are more prevalent in trees where wetting is more easily accomplished. A detailed analysis of ions is needed in Douglas-fir tissue undergoing decay as has been done in balsam fir (Shortle and Bauch, 1986), red spruce (Shortle and Minocha, 1990), and red maple (Shevenell and Shortle, 1986).

A significant decrease in soluble phenol concentration was observed in the previsual stage of whiterot (Table 6). A significant decrease in the soluble phenolic substances of wood undergoing whiterot is a consistent feature of this type of decay (Tattar et al., 1971; Tatter and Rich, 1973; Smith et al., 1981; Shortle and Smith, 1987). The production

Table 9. Electrical Resistance of Water Extracts and Mild Alkali (1% NaOH) Solubility of Douglas-fir Wood Taken From a Utility Pole With Visible Brownrot.

	Distance from visible decay, cm[a]			
Tissue	0	6	12	17
	----------R_e, k ----------			
Inner heartwood	<u>25</u>*	27*	39	47
Outer heartwood	32*	47	54	57
Preserved sapwood	50	46	59	59
	---------alkali solubility, %----			
Inner heartwood	<u>52</u>	30	19	19
Outer heartwood	20	18	-	19
Preserved sapwood	15	15	-	15

[a]Value for visibly decayed sample underlined, other samples were either sound or in previsual stages of decay. R_e - mean extract electrical resistance (0.2 mg wood/5 mL H_2O), two observations per mean, asterisk (*) indicates mean differs significantly from the mean for preserved sapwood ($P < 0.01$). Alkali solubility (1% NaOH) based on single observation.

of extracellular phenol oxidases that favor polymerization of polyphenols is a common feature of whiterot fungi and effects the growth of these fungi (Taylor et al., 1988a, 1988b, and 1989). The depolymerization of lignin by whiterot fungi would not be favored as long as a high concentration of polyphenol was favoring a polymerization reaction (Kirk and Farrell, 1987).

Brownrot fungi do not produce extracellular phenol oxidases and an increased concentration of phenols were observed in tissue undergoing decay by brownrot fungi after an initial decrease (Table 6). The source of increased phenol concentration is not yet known. The increased concentration may be due to changes in the wall polymers of wood or to products of the brownrot fungus itself.

A significant increase in mild alkali solubility occurred during the late previsual stage of brownrot (Table 7). Increased mild alkali solubility of wood has been associated with a rapid change in the degree of polymerization of wall polymers (Cowling, 1961). Decreased extract electrical resistance characteristic of all stages of brownrot from early previsual to visual and increased mild alkali solubility characteristics of late previsual brownrot were both observed in tissues contiguous with visual brownrot in a decayed Douglas-fir utility pole (Table 9).

A significant decrease in extract electrical resistance was a consistent feature of the early previsual stage of whiterot and brownrot in a variety of wood species (Table 8). Low pH of the extract gives some indication of brownrot, but this may vary with species. A significant decrease in phenol concentration characteristic of whiterot and a significant increase in phenol concentration characteristic of brownrot is not consistently associated with the early previsual stage of decay, but may occur during the late previsual stage depending on tree species studied.

Decay of wood in trees and in service is a dynamic process in which the previsual stages need to be better understood so that better methods of detection, diagnosis, and preservation can be developed (Smith, 1987 and 1989). Once decay has reached the visual stage, wood properties have become drastically altered and remedial action is difficult. Treatments that interfere with processes such as ion accumulation and phenol reduction in the previsual stages need to be considered as means of improving wood preservation.

SUMMARY

Early stages of wood decay involve the rapid accumulation of ions in wood during the first 10 days of incubation of sapwood or heartwood with a brownrot fungus, Postia placenta, or a whiterot fungus, Coriolus

versicolor. Rapid ionization of wood by the brownrot fungus involved acidification followed by increased phenol content and increased alkali (1% NaHO) solubility prior to visual decay detection. The ionization of wood by the whiterot fungus initially was slower than by the brownrot fungus. Ionization of wood by the whiterot fungus involved the accumulation of soluble potassium salts, rather than acids, as in the case of brownrot. Phenol content decreased, and alkali solubility increased slightly prior to visual detection of whiterot. The same general pattern of ionization was observed in sapwood, and heartwood of several species of softwoods (Douglas-fir, red pine, and red cedar) and hardwoods (red oak and black cherry), although rates of change varied. Ionization of wood can be detected by measuring changes in electrical resistance. Treatments to retard the accumulation of ions are likely to improve decay resistance of wood. Thus, a better understanding of the ionization processes may lead to improved methods of detecting and preventing the biodeterioration of wood.

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Changes in Cell Wall Components of White Pine and Maple by White-Rot Fungi

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INTRODUCTION

Few detailed studies have been made of the relative rates of removal of the structural components of wood (cellulose, hemicelluloses, and lignin) during decay by white-rot fungi. Kretsberg et al. (1971) showed that the total pentosans are destroyed faster than the cellulose, and the lignin more slowly than cellulose or pentosans, during the decay of spruce by the white-rot fungus Trametes trogii. Kirk and Highley (1973) found that the relative rates of removal of lignin and the other components by three white-rot fungi in conifer woods decayed in soil-block tests varied during the decay process. Their results suggested that removal of glucomannan may precede removal of cellulose as found in brown-rots, but that additional wood and fungus combinations are needed before it can be established whether this is a valid generalization for white-rots. For hardwoods, such detailed analysis seem to have been done only by Cowling (1961) who found that Coriolus versicolor removed lignin and carbohydrates at about the same rate in sweetgum. Removal of glucan, mannan, and xylan, however, was not determined until 25% weight loss.

The selective removal of cell wall components by white-rot fungi may be changed by nutritional factors that influence fungus physiology. For example, Blanchette et al. (1985) found that Ganoderma applanatum and Ischnoderma resinosum selectively delignified wood in nature, but in

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laboratory soil-block tests, all cell wall components were removed. Factors that affect cellulose, hemicellulose, and lignin degradation need to be identified. One such factor appears to be nitrogen. Low nitrogen levels enhanced degradation of lignin model compounds by P. chrysosporum (Kirk et al., 1978). Later studies showed that low nitrogen induced formation of H_2O_2 and ligninase in P. chrysosporium. On the other hand, high carbohydrate levels repressed carbohydrate and lignin degrading enzymes of P. chrysosporium (Ericksson, 1978; Kelly and Reddy, 1986).

It is important to establish if certain cell wall components are preferentially removed during decay and to determine if cultural parameters, such as nitrogen and carbohydrate levels, govern selectivity of removal. For example, if hemicellulose and/or lignin utilization are essential early steps in the decay process, blocking these steps will stop the whole decay process. The specific purposes of obtaining these data are (1) to elaborate on the changes in individual cell wall components during decay by selected white-rot fungi in hardwoods and soft woods, and (2) to determine the effect of exogenous nutrients on removal of cell wall constituents.

MATERIALS AND METHODS

Wood Samples and Decay Tests

Sapwood blocks 6.35 by 6.35 by 3.18 mm (1/4 by 1/4 by 1/8 in., the small dimension in the fiber direction) were cut from western white pine (Pinus monticola Dougl.) and maple (Acer spp.). Small test blocks were used rather than large blocks because small blocks provide more uniform decay throughout, particularly in the early stages. The blocks were numbered, conditioned to constant weight at 27°C and 70% relative humidity, and then weighed. Blocks were decayed by the American Society for Testing and Materials Standard soil-block method (ASTM, 1971) and an agar-block method. The soil-block method involves contact of the test wood with actively growing fungus on wood in soil contact, which provides a source of nutrients for the fungi, thus enhancing decay. The agar-block tests used in this study were designed to provide favorable conditions for decay, but also to prevent contamination of the block by foreign nutrient material and

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leaching of degradation products from blocks. With this method, the test fungi were grown on Whatman[2] No. 1 filter paper strips placed over a nutrient-agar medium (Highley, 1973a). After the fungi covered the filter paper, the strips were removed and placed on triangular-shaped glass rods over 1.5% water agar containing no additional nutrients, 10% glucose, 0.5% NH_4NO_3 or 10% glucose plus 0.5% NH_4NO_3 in 8-oz French square bottles. In both soil-block and agar-block chambers, 20 blocks per bottle were decayed at different lengths of time to obtain samples in various stages of decay. Following incubation, the blocks were removed, reconditioned, weighed, and their weight losses calculated. Noninoculated blocks served as controls.

Fungual Culture

Blocks were decayed by the following white-rot fungi: Phanerochaete chrysosporium Burds. (ME-461), Coriolus versicolor (L.ex Fr.) (MAD-647), Irpex lacteus (Fr.:Fr.)Fr. (HHB-7328-sp.), Bjerkandera adusta (Willd.:Fr.) Karst. (L-15359-sp.), and Phlebia brevispora Nakas. in Nakasone et Eslyn (HHB-7030-sp.).

Analytical Analysis

Sound and decayed wood blocks were ground to pass a 0.50-mm (40-mesh) screen, and the meal dried thoroughly at 45°C under high vacuum. The samples were analyzed for Klason lignin using previously described methods (Effland, 1977). Relative amounts of glucose, xylose, and mannose in acid hydrolysates were determined using high-pressure liquid chromatography as described by Petterson et al. (1984). From these values the glucan, xylan, and mannan, and losses of each during decay were calculated (Springer, 1966).

Cellulose (~41%), hemicelluloses (~26%), and lignin (~29%) are the principal components of conifer wood. The two principal hemicelluloses are (1) a galactoglucomannan (~16%), which is about 70% mannan, and (2) an arabino-4-O-methylglucuronoxylan (~10%), which is about 65% xylan (Timmell, 1967). Thus, the measured amount of glucan is an estimate of cellulose content with a small error due to glucomannan; the amount of mannan is an estimate of the major hemicellulose, and the amount of xylan an estimate of the minor hemicellulose.

In angiosperm wood, O-acetyl-methylglucuronoxylan is the major hemicellulose (~25%), which is about 75% xylan. The minor amount is of the glucomannan type (~3%), which is about 65% mannan.

RESULTS

The analytical values for the wood decayed over soil or water-agar are

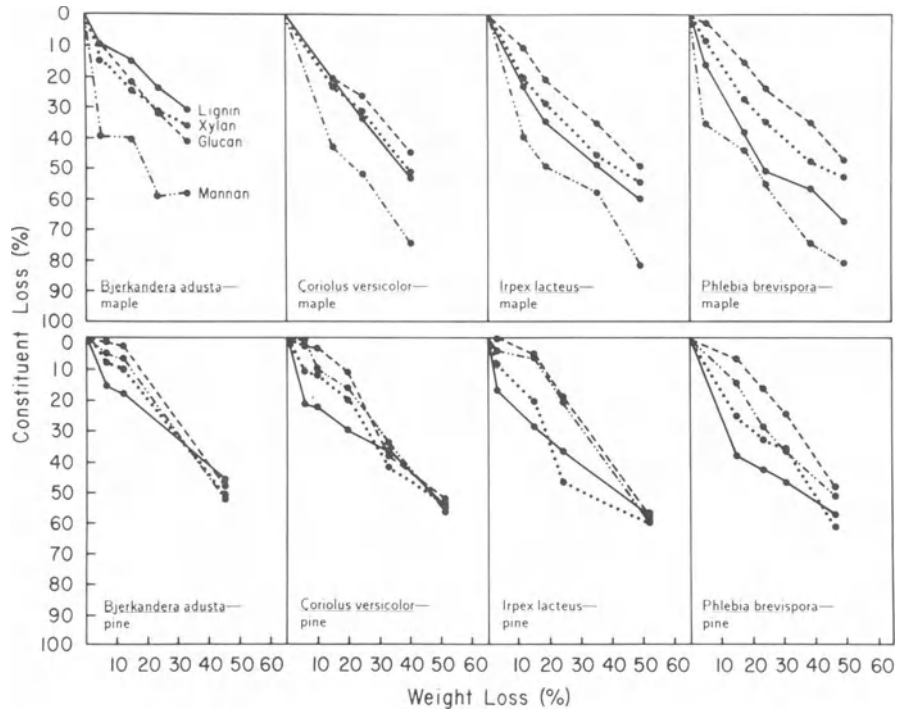

Figure 1. Progressive Loss of Major Structural Components in Pine and Maple Decayed by Four White-Rot Fungi by the Soil-Block Method. At Least 20 Blocks of Similar Weight Loss Were Combined for Analysis. Data are Based on a Single Determination of Each Component in the Combined Blocks and are Expressed on the Basis of the Original Amount of Each Component in the Sound Wood. The Analytical Methods have Proven to Give Reproducible Values (Moore and Johnson, 1967).

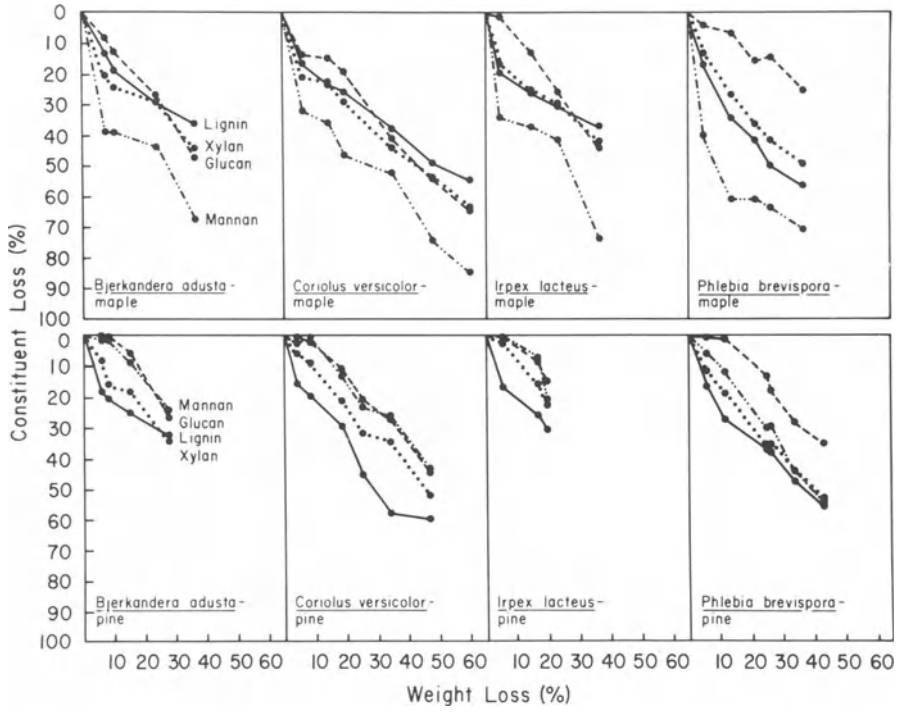

Figure 2. Progressive Loss of Major Structural Components in Pine and Maple Decayed by Four White-Rot Fungi by the Agar-Block Method. See Figure 1 for Explanation of Data.

illustrated graphically in Figures 1 and 2 as loss in lignin, glucan, mannan, and xylan, with results expressed as a percentage of the original amounts of each.

On the same wood species, the relative rate of removal of the major structrual components was similar for all the white-rot fungi. Likewise, the relative rate of removal of components in wood decayed over soil was similar to that decayed over water-agar. Removal of components did differ with the wood species. In white pine, at the lower weight losses, lignin was removed faster than the carbohydrate components. As decay progressed, amount of lignin removal was generally comparable to the other components. In maple, lignin removal usually progressed at a rate similar to or slightly faster than xylan and glucan. Mannan was removed at a substantially faster rate in maple than all the other components by all the fungi.

Table 1 compares the loss in weight of maple blocks exposed over water-agar to that obtained with glucose or NH_4NO_3 added to the medium. Ten percent glucose caused marked inhibition of decay by all the fungi and completely stopped decay of Ph. brevispora and B. adusta over the 24-month incubation period. Ammonium nitrate (0.5%) had little effect on the decay rate of the fungi except for Ph. brevispora where decay decreased. Glucose and NH_4NO_3 together decreased the rate of decay about the same as glucose alone.

The effect of glucose and NH_4NO_3 on removal of cell wall components in maple by the white-rot fungi is given in Table 2. Where decay occurred, glucose stopped cellulose utilization and accelerated lignin utilization. With NH_4NO_3 in the medium, removal of cell wall components from maple differed little from blocks exposed over water agar. Similarly, glucose and NH_4NO_3 together had little effect on removal of cell wall components where decay occurred.

DISCUSSION

The relative rates of removal of the major structural components in pine and maple among the white-rot fungi were similar. However, the fungi did remove cell wall constituents at a different rate in white pine than in maple. Differences in removal of cell wall constituents in different woods by the same white-rot fungus have been observed in other studies (Kirk and Highley, 1973; Kirk and Moore, 1972). Brown-rot fungi, on the other hand, remove cell wall components in wood at about the same rate in different wood species (Highley, 1987) and remove mannan substantially faster than the other cell wall components. Similarly, the white-rotters in this study preferentially removed mannan in maple. However, lignin was removed faster

Table 1. Effect of Glucose and NH_4NO_3 on Weight Loss by White-Rot Fungi in Maple[a].

	Weight loss (%)															
	C. versicolor				*Ph. brevisporia*				*Irpex lacteus*				*B. adustus*			
Time (weeks)	W-A	Gl	N	Gl+N	W-A	Gl	N	Gl+N	W-A	Gl	N	Gl+N	W-A	Gl	N	Gl+N
2	6	0	7	0	0	0	0	0	0	0	0	0	3	0	0	0
4	11	0	17	0	9	0	0	0	6	0	5	0	5	0	5	0
8	48	4	30	0	22	0	0	0	23	0	16	0	15	0	12	0
10	49	10	41	8	28	0	9	0	25	0	35	0	16	0	15	0
16	-	11	-	12	-	0	15	0	-	0	37	0	-	0	30	4
24	-	12	-	18	-	0	17	0	-	14	41	5	-	0	36	11

[a] W-A, 1.5% agar in water medium
Gl, 10% glucose added to agar medium
N, 0.5% NH_4NO_3 added to agar medium

Table 2. Effect of Glucose and NH_4NO_3 on Loss of Major Structural Components from Maple Decayed by White-Rot Fungi.[a]

Fungus, nutrient	Time	Loss (%) Total weight	Lignin	Glucose	Mannan	Xylan
C. versicolor						
Glucose	10	6	8	0	3	7
	16	10	34	0	7	14
	24	17	44	0	10	28
Nitrogen	2	6	7	3	10	8
	4	17	21	17	20	18
	8	28	31	22	40	27
	16	48	45	53	63	46
Glucose + Nitrogen	10	5	3	0	4	4
	12	8	7	7	7	7
	24	18	21	15	10	18
	24	25	32	19	15	28
Irpex lacteus						
Glucose	24	9	44	0	13	19
	24	15	51	0	13	25
Nitrogen	4	8	8	8	20	8
	8	16	14	23	37	17
	10	24	21	35	50	25
	16	35	29	45	53	37
	24	46	49	52	50	48
Glucose + Nitrogen	24	5	3	5	0	6
	24	7	6	6	0	7
Bjerkandera adustus						
Glucose	24	0	0	0	0	0
Nitrogen	4	5	5	6	0	7
	8	10	12	8	3	5
	10	15	16	17	20	14
	12	23	23	26	27	20
	16	33	30	40	50	30
	24	44	39	49	63	38
Glucose + Nitrogen	16	6	0	4	13	4
	24	11	19	8	13	13
	24	15	23	15	17	13
Phlebia brevispora						
Glucose	24	0	0	0	0	0
Nitrogen	16	9	6	2	3	8
	24	15	20	6	10	28
	24	17	37	5	20	28
Glucose + Nitrogen	24	0	0	0	0	0

[a]Blocks were exposed to decay fungi over an agar medium with 10% glucose, 0.5% NH_4NO_3 or 10% glucose plus 0.5 NH_4NO_3.

in white pine than the other components. In another study (Highley, 1982), C. versicolor removed carbohydrates faster than lignin from western hemlock, white spruce, and southern pine early in the decay process. As decay progressed, lignin and carbohydrates were removed at about the same rate. In this study, the white-rotters removed the hemicellulose faster than cellulose in both maple and white pine. As with brown-rotters (Highley, 1987), removal of cell wall constituents by the white rotters was similar when decayed by the soil-block or the low nutrient agar-block method.

The ability of white-rotters to degrade cell-wall components of wood is dependent upon production of serveral extracellular enzymes. Lignin degradation is dependent upon a number of enzymes collectively called ligninases, and carbohydrates are degradated by cellulase and hemicellulase complexes. Nitrogen supply plays a crucial regulatory role in wood decay in that high nitrogen levels may inhibit formation of ligninolytic enzymes and lignin degradation by white-rot fungi (Kirk, et al., 1978). On the other hand, nitrogen levels are reported to have a variable effect on cellulose decomposition by fungi (Park, 1976). Some fungi decomposed cellulose more slowly with low nitrogen availability, while others decomposed cellulose more slowly with increased nitrogen concentrations (Park, 1976). High nitrogen levels have been found to stimulate carbohydrate breakdown by some wood decay fungi (Levi and Cowling, 1969; Reid, 1983). Dill and Kraepelin (1986) found that adding nitrogen sources resulted in rapid and almost complete decay of the cellulose in Palo padrido. In the present study, high nitrogen levels in the medium did not affect rate of decay or lignin or carbohydrate utilization.

Glucose in the medium drastically reduced the rate of decay in maple by all of the white-rotters. Petterson and Cowling (1964) investigated the resistance of Sitka spruce and southern pine woods to attack by white-rot fungi. They demonstrated that the resistance of the wood was reduced by impregnating it with a 1% solution of glucose. In another study, Darbyshire et al. (1969) found that high glucose levels in the medium decreased decay rate in apple wood by Trametes (=Coriolus) versicolor. Most likely the reduction in wood decay by white-rotters associated with high sugar levels is related to failure to produce enzymes responsible for carbohydrate breakdown. Simple sugars have been found to repress production of both cellulases and hemicellulases by white-rot fungi (Ericksson and Goodell, 1974; Highley, 1973b, 1976). Where decay in maple occurred in the presence of glucose, removal of cellulose was completely inhibited. Lignin degradation in maple was accelerated over the

glucose medium even though glucose is reported to repress ligninolytic enzymes of white-rot fungi (Kelly and Reddy, 1986).

With respect to susceptiblity of wood to decay by white-rot fungi, our results suggest that if a continuous and relatively high level of simple sugar could be maintained in wood, decay would be inhibited. This, of course, would be a very impractical method of wood protection. However, if nonmetabolizable sugar analogs could be found that repress decay, it might offer an opportunity to protect wood in an environmentally safe manner.

SUMMARY

Miniature blocks of white pine and maple wood were decayed by four white-rot fungi: Coriolus versicolor, Irpex lacteus, Bjerkandera adusta, and Phlebia brevispora. The blocks were decayed over a soil medium, a low-nutrient agar medium, and an agar medium supplemented with 10% glucose, 0.5 NH_4NO_3 or 10% glucose plus 0.5 NH_4NO_3. Quantitative changes in lignin, glucan, mannan, and xylan during decay were determined. On the same wood, white-rot removal of the cell components was similar. Removal of components in white pine differed from that in maple. In white pine, lignin was removed faster than the carbohydrates. In maple, mannan was removed substantially faster than the other components. Glucose caused a marked inhibition of decay, while NH_4NO_3 had little effect. Glucose stopped cellulose utilization and accelerated lignin utilization. NH_4NO_3 had little effect on relative rate of removal of cell wall components.

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Immunological Characterization of Fungal Enzymes and Biological Chelators Involved in Lignocellulose Degradation

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INTRODUCTION

Understanding the basic mechanisms underlying wood decay is currently of interest because of the need for decay prevention in in-service wooden structures. Additional interest stems from the potential use of biological processes to modify or alter wood, and thereby produce useful chemicals, fuels, pulp, or other materials. Although cellulose and lignin (the basic components of wood) are the two most abundant organic materials on earth, still little is known about the mechanisms involved in their degradation. It has long been postulated that enzymes are involved in the decay process, but the first ligninase from a white-rot fungus was isolated for characterization only as recently as 1983 (Tien and Kirk). More attention has been focused on this field in the last six years but still little is known about enzymes and other metabolites from wood degrading fungi. This is particularly true with regard to how, and if, both cellulose and lignin destroying enzymes penetrate into the wood substance to cause degradation.

One current hypothesis is that it is not possible for enzymes alone to penetrate into the wood cell wall to cause degradation. This is because the micropores (sub-microscopic size holes, known to exist in the wood cell wall) are not large enough to allow enzyme penetration. To provide for enzyme penetration, a non-enzymatic system involving metal ions (iron or perhaps manganese or copper), and a radical generating system may be involved (Highley, 1980; Koenigs, 1974; Murmanis et al., 1988; Kirk, 1988). This system, still under study by many researchers, is thought to operate as an initial decay step within the cell wall, opening up the pores to allow the

enzymes to penetrate the microstructure. Degradation patterns differ between different types of decay fungi; however, non-enzymatic degradation systems have been proposed for both of the two basic types of decay; brown-rot and white-rot (Srebotnik and Messner, 1988; Srebotnik et al., 1988).

Opposing data (Schmuck et al., 1986) have been presented suggesting that some cellulosic degrading enzymes from other types of fungi are of small enough molecular weight (<12,000 M.W.), and shaped long and thin, so that they could penetrate the wood pores. Other evidence suggests that enzymes could be produced in a deglycosylated but still functional form (Micales and Highley, 1988). It is possible that this smaller enzyme form might be able to penetrate the wood cell wall to cause limited degradation. Still other workers suggest that "large" micropores could exist in some plant cell walls to permit the entry of molecules as large as 60,000 MW (Tepfer and Taylor, 1979; Carpita et al., 1979).

Using techniques developed in our laboratory and in collaboration with other groups, we have made a start towards exploring the enzymatic versus non-enzymatic degradation hypotheses. The approach that we have developed in our laboratory to study the degradative mechanisms involved in the fungal attack of wood has been the use of immunological (Goodell and Jellison, 1986; Jellison and Goodell, 1986: Jellison and Goodell, 1988; Goodell et al., 1989) and cytochemical probes for the labelling of fungal enzymes and other metabolites in the woody substrate. In this paper we will review recent advances in our understanding of the enzymatic and non-enzymatic degradation of wood as understood through the use of tools such as immuno-TEM, ELISA (enzyme-linked immunosorbent assay), and other immunological techniques. We also propose the involvement of another metabolic agent, fungal siderophores, in the biological degradation of wood by higher fungi.

DEGRADATIVE MECHANISMS

Two basic types of wood biodegradation will be considered in this paper, brown and white rot. Brown-rot decay fungi primarily degrade cellulose and hemicellulose, often degrading cellulose in a process similar to acid hydrolysis, at a rate in excess of what they can utilize. An enzyme capable of duplicating these effects in wood has never been isolated from a brown-rot fungus and cellulase enzymes thus far isolated are too big to diffuse freely into the wood microstructure. Brown-rots produce a constitutive enzyme complex with endo-1,4-β-D-glucanase activity, but many brown-rot fungi apparently lack exo-β-1,4-glucan cellobiohydrolase activity (Kirk and Cowling,

1984). A multi-enzyme complex has been isolated from Postia placenta and shown to hydrolyze xylan, mannan and carboxymethyl cellulose (Wolter et al., 1980). Involvement of a nonprotein hydrogen peroxide/iron system via an oxidative mechanism has been suggested as an explanation for the ability of brown-rot fungi to vigorously degrade wood cellulose in the absence of detectable exo-1,4-β-glucanase activity (Highley, 1980; Koenigs, 1974). Recently electron spin resonance (ESR) has been used to detect hydroxyl radicals in liquid cultures and wood samples inoculated with the brown-rot P. placenta (Illman et al., 1988b). ESR has also been used to identify changes in paramagnetic resonance in wood during brown-rot decay (Illman et al., 1988a). The lignin framework remaining after carbohydrate removal by brown-rot fungi is still polymeric but is characterized by an increased alpha-carbonyl and carboxyl content, demethylation of methoxyl groups and increased phenolic hydroxyl content. Even though extensive depolymerization of the lignin does not occur, limited oxidation of aromatic and propyl side chain carbons has been demonstrated (Kirk and Cowling, 1984).

White-rot fungi produce endo-1,4-β-glucanases, exo-1,4-β-glucanases and β-glucosidases which act synergistically to degrade cellulose (Shoemaker, 1985), and a non-enzymatic depolymerizing agent also appears to play a role in white-rot degradation. Ultrastructural changes caused by white-rot fungi attacking isolated cellulose fibers appear similar to those caused by brown-rot fungi. Further insight into cellulose and hemicellulose degradation by both brown and white fungi can be obtained from numerous recent studies on the filamentous fungi (Enari, 1985; Shoemaker, 1985; Warren, 1986; Knowles et al., 1987).

Lignin can be completely metabolized to CO_2 by white-rotting fungi. Several enzymes are involved in lignin breakdown including lignin peroxidase, manganese peroxidase, laccases, and oxidase. Lignin peroxidase catalyzes the H_2O_2 dependent oxidation of lignin. Substrates can be oxidized by 1 or 2 electrons. The main isozyme form of the enzyme is a glycoprotein with a molecular weight of 41,000 daltons. The enzyme is of broad specificity, and is capable of causing the partial depolymerization of lignin. Since the isolation of the first ligninase, other enzymes capable of participating in lignin break-down have been isolated with specific lignin peroxidases isolated in multiple forms (Kirk et al., 1986). Renganathan et al. in 1985 reported three molecular forms of diarylpropane oxidase produced by Phanerochaete chrysosporium. All ligninases examined have a single iron protoporphyrin IX prosthetic group. A series of water soluble, sterically stablized porphyrins have been used to mimic ligninase activity (Dolphin, 1986; Dolphin et al.,

1987). Paszczynski et al., 1988, have examined the delignification of wood using natural and synthetic porphyrins. The molecular biology of the ligninase gene is being examined by numerous laboratory groups (Tien and Tu, 1987; Broda et al., 1987; Sims and Broda, 1987; Farrell et al., 1987). Another ligninase, MnII-dependent peroxidase, has also been isolated (Glenn and Gold, 1985). This enzyme is a 46,000 MW glycoprotein with a heme prosthetic group and is dependent on both H_2O_2 and MnII for activity. Unlike other extracellular ligninases the MnII peroxidase appears to be associated closely with the fungal hyphae (Paszczynski et al., 1986).

ULTRASTRUCTURE, IMMUNOLOGICAL, AND GOLD PROBE ANALYSES FOR MECHANISM ELUCIDATION

Antibodies have previously been used to elucidate the mechanisms of lignocellulosic degradation in our own lab (Jellison and Goodell, 1986). Other studies have been done including Kirk and Tien, 1986, who used polyclonal western blot analysis to reveal a cross-reaction among three ligninases. Farrel et al., 1987, have shown that cloned ligninase isozyme was recognized by antibodies against native isozymes H_2 and H_8. Wood et al., 1987, have used antibodies to differentiate two cellobiohydrolases. Morgenstern et al., 1987, have used monoclonal antibodies (Mabs) to examine cellulolytic glycoconjugates from Clostridium thermocellum. Cellulase Mabs (made to the thermophilic fungus Talaromyces) have been used by McHale, 1987, to identify an antibody specific for endoglucanase and to demonstrate immunological differences in the forms of endoglucanases. Messner and Kubicek, 1987, have used polyclonal antibodies to help identify endo-β-1,4 glucanase precursers.

Messner et al., 1987, used colloidal gold labeled polyclonal antibodies to show ligninases located preferentially on the plasmalemma of fungal hyphae. Concentration of ligninases on outer surface of hyphae wall was much lower, (possibly due to the preparatory methods used). However, because of the heterogeneous nature of polyclonal antibodies, and the potential similarity of glycosylated sequences on some enzymes, interpretation in localization studies using polyclonal antibodies to glycosylated fungal metabolites can be difficult. Srebotnik and Messner, 1988, used antibodies to lignin peroxidase to localize the enzyme in wood specimens infiltrated with concentrated culture filtrates. Leisola et al., 1987, used transmission electron microscopy to study ultrastructural aspects of wood decay. No labelling was observed after incubation of thin sections with ligninase-gold complexes and wood partially

hydrolyzed by ligninolytic fungi was not labeled by the enzyme probe. All white rotters tested, except P. ostreatus, were labeled with anti-ligninase-protein A gold. The ligninase probe was located near the fungal plasmalemma and was not seen extracellularly. Garcia et al., 1987a,b, studied the binding of ligninase peroxidase-gold probes to wood. Native wood was not labelled but the probe was observed intracellularly in cytoplasmic areas close to plasmalemma in P. chrysosporium and sometimes in fungal slime. No extracellular lignin peroxidase was detected in the proximity of the fungus or in association with the wood. Absence of wood labeling with a ligninase-anti-ligninase-protein A gold probe was postulated to be due to inaccessibility of wood lignin to the enzyme active site. Using 3,3'-diaminobenzidine (DAB), peroxidases were detected extracellularly on the fungal cell wall surface and on wood in areas of degradation. Previous work (Forney et al., 1982) had shown evidence for peroxidatic activity within the periplasmic space.

More recent immunolabelling studies (Daniel et al., 1988) have shown ligninase closely associated with both P. chrysosporium and exposed sites of erosion decay of wood fibers. TEM studies showed labelling on all cell wall layers. Cytochemical studies with DAB and other peroxidase indicators suggested an intracellular distribution within the S_2 cell wall of the degrading fibers (Goodell et al., [Figure 1], 1989; Daniel et al., 1988). Analysis of Phanerochaete chrysosporium degraded wood, probed with a Mab to Mn-dependent peroxidase has shown localization of this enzyme within the middle lamellae when this site was exposed to attack by wall fracture or severe degradation (Goodell et al., 1989) (Figure 2). Mn-dependent peroxidase was also localized within cell wall regions undergoing degradation. Blanchette et al. (personal communication, in press, 1989a,b) have worked with a number of direct conjugate probes and with mono- and polyclonal antibodies produced to ligninases, cellulases, and xylanase. In white-rotted wood, ligninase was found localized in heavily degraded cell wall material, and also in degraded middle lamellae. Xylanase and cellulase were found throughout the wood cell wall after fungal attack, in both brown- and white-rotted wood but this varied with the stage of attack and depletion of specific substrates in the wall.

It is not known at present how these enzymes of relatively high molecular weight can move into the wood cell wall and middle lamellae. As previously discussed, it has been hypothesized that low-molecular-weight, non-enzymatic materials may move into the wood cell wall ahead of enzymes, opening up the pore structure of the wood to provide greater enzyme access. To date,

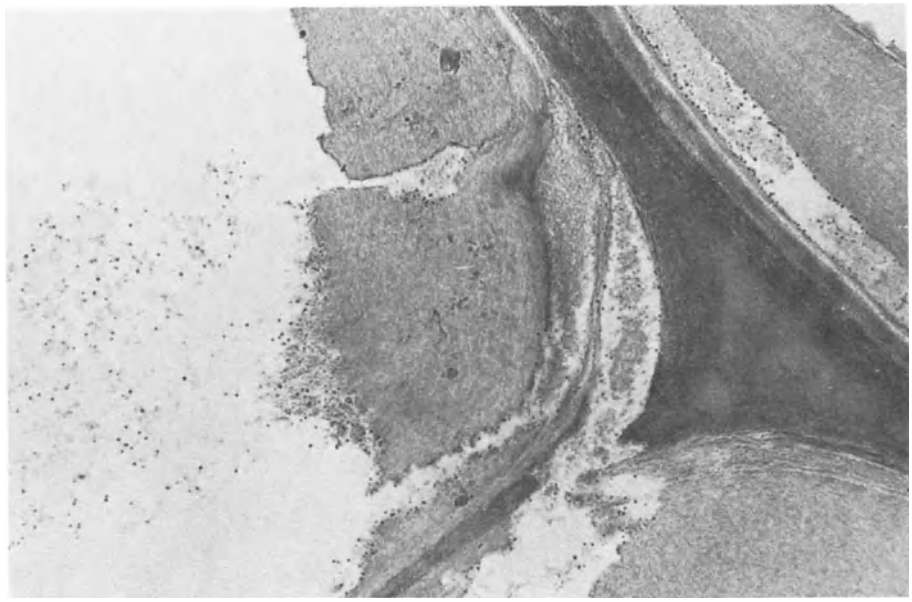

Figure 1. Peroxidase-Indicating Reaction Product Showing Labelling in the Extracellular Fungal Slime, on the Surface of Degraded Cell Wall Material, and Within the S2 Layer of the Wood Cell Wall in Severely Degraded Cell Wall Material.

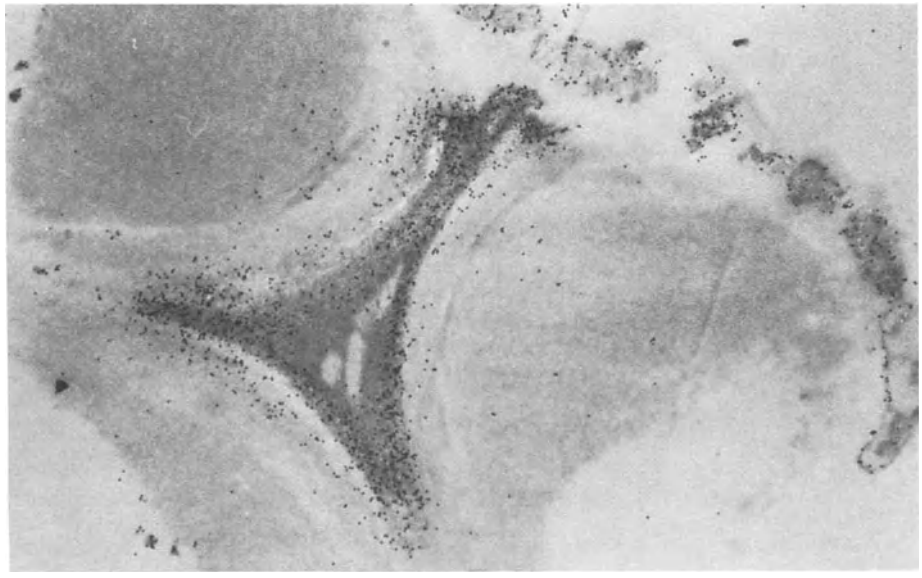

Figure 2. Mab Peroxidase Labelling of Extracellular Slime and Degraded Wood Material. Intense labelling of the exposed portions of the middle lamellae is noted. Labelling is less intense toward the less degraded region of (central) cell corner/ middle lamellae region.

although several hypothetical non-enzymatic degradative systems have been proposed (as previously outlined), no non-enzymatic system capable of degrading lignin or cellulosic components has been proven functional in an intact hymenomycete/lignocellulose system.

BIOLOGICAL CHELATORS PRODUCED BY DEGRADATIVE FUNGI

Iron, and other metals such as manganese, play a role in biological degradation as essential elements for fungal metabolism and growth. In addition, iron plays a role in biodegradation both 1) as a component of the extracellular heme enzymes involved in white-rot degradation (Paszczynski et al., 1988; Tien and Kirk, 1983), and 2) possibly in brown-rot organisms in a non-enzymatic iron/hydrogen peroxide catalysis of cellulose degradation (Highley, 1980; Koenigs, 1974; Murmanis et al., 1988).

To solubilize and sequester ferric iron, many fungi utilize high affinity iron acquisition systems (Neilands, 1974; Neilands et al., 1987; Hider, 1984) mediated by siderophores. Siderophores are low molecular weight (500-1000), primarily ferric-specific ligands, whose biosynthesis is regulated by iron level. They are selective metal chelators and their biosynthesis is regulated by iron levels.

The ability of selected basidiomycetous decay fungi to produce siderophores has been demonstrated (Fekete et al, 1989) using a modification of the Chrome Azurol-S assay (Schwyn and Neilands, 1987) and a rapid paper electrophoresis assay (Fekete et al., 1983). Different microorganisms are known to produce at least two chemical types of ligands: the secondary hydroxamic acids and the phenolates. In the paper electrophoresis assay phenolates are detected by fluorescence under UV light. Hydroxamic acids are visualized by spraying with ferric acid. The compounds isolated from the decay fungi tested appear to be phenolate in character. Evidence of biological chelators being produced was obtained for ten degradative fungi with some organisms, such as Coriolus (Trametes) versicolor, showing evidence of producing up to three forms of iron chelating metabolites.

Siderophores have been isolated from Gloeophyllum trabeum (brown-rot) and Coriolus versicolor (white-rot). Cultures were grown on iron-deficient media and siderophores were purified via rotary evaporation of culture media followed by Amicon ultrafiltration using a YM2 membrane to isolate metabolites of MW 1000 or less. This crude fraction was then subjected to ethyl acetate precipitation (Westervelt et al., 1985) and purified by thin layer

chromatography in a solvent system of 40:40:10:10 chloroform:etylacetate: formic acid:toluene. A liquid Chrome Azurol-S assay was used to monitor the purification. The iron-repressibility of the chelators has been demonstrated using the white-rot fungus Coriolus versicolor (Fekete et al., 1989) and the brown-rot fungus Gloeophyllum trabeum (Jellison and Chandhoke, unpublished).

Experiments by Dolphin, 1986, Dolphin et al., 1987, and Paszczynski et al., 1988, which demonstrated activity of different porphyrins and non-biological chelators on lignin, suggest the possibility of direct involvement of siderophores in the process of wood degradation. To test the potential of siderophores in carrying out non-enzymatic wood modification, we have performed preliminary experiments using two siderophores (putatively phenolate type) isolated from decay fungi. One and one-half milliliters of ethyl acetate siderophore extract (Westervelt et al., 1985) was added to 2 g (O.D.) of unbleached red spruce pulp and incubated for four hours. Within 30 minutes a bleaching effect on the pulp was apparent (Figure 3) suggesting action of the siderophores on the chromophoric groups of the pulp.

FUTURE WORK

The production of siderophores by hymenomycetous fungi has only recently been reported. As such, there are many unknowns that remain to be answered with regard to siderophore function in these fungi. However, because our preliminary studies suggest that these metabolites can bleach chromophoric groups in unbleached pulp, and similar low molecular weight chelators have been shown to perform similar oxidation/reduction reactions, this suggests that biological chelators may play a role in lignocellulose breakdown. Questions of potential interest include:

--If siderophores are involved in biological degradation, why is there a selective attack of cellulose and hemicellulose, but not lignin in brown-rot? Demethoxylation (in lignin) and other reactions occur, but attack of the lignin ring does not. To help answer this question, immuno-localization of siderophores and enzymes in degraded wood and degraded cellulose and lignin plus the biochemistry and chemistry of these metabolites must be studied. For example, Popp and Kirk (unpublished, Raleigh, 1989) have suggested that some non-siderophore chelators do not have the oxidative potential to carry out lignin degradation. Does the oxidative potential of brown-rot siderophores differ from that of white-rot siderophores?

--Why does rapid depolymerization of cellulose occur in brown-rot (but not white-rots) in very early decay stages and throughout the wood cell wall? In white-rots (classical white rot) only lumen

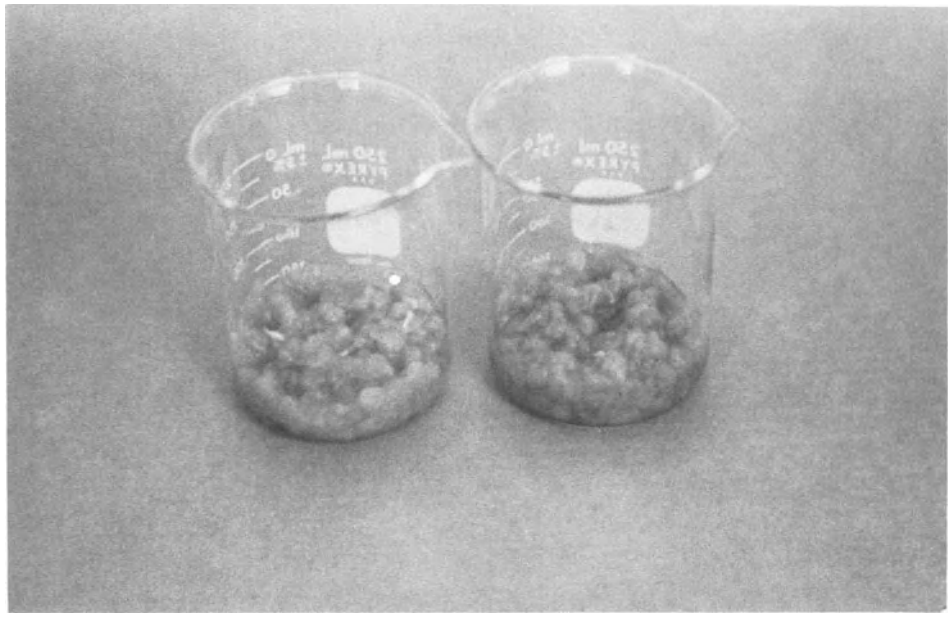

Figure 3. Brightening Effect of Siderophores Isolated from Decay Fungi in Unbleached Pulp Fibers. Siderophore treated pulp (1/2 hour treatment, unwashed) on left. Control unbleached pulp on right.

surface attack of the wood generally occurs and the cell wall is gradually eroded away. Immunolabelling studies could show how enzymes and siderophores differ in distribution across the degraded wood cell wall, allowing distinctions in the pattern of degradation in brown- and white-rotted wood to be made.

--If siderophores play a significant role in biological degradation, why are only brown-and white-rot basidiomycetes the most aggressive in breaking down the wood cell wall? Some non-decay fungi have been shown to produce hydroxamate siderophores whereas preliminary evidence suggest phenolate-type siderophores may be the predominant form produced by decay fungi. Is the form of siderophore produced a factor in an organism's ability to degrade wood, even when that organism has been shown to produce degradative enzymes? Some fungi such as Trichoderma produce cellulases and other degradative enzymes, but do not aggressively attack the wood cell wall. (Trichoderma has not previously been reported to produce any form of siderophore.) Could this help to explain the action of some non-

decay species that otherwise have the enzymatic potential to attack at least some constituents of the wood cell wall?

SUMMARY

A condensed review of the literature pertaining to immunolocalization of enzymes in the decay process has been provided. Although reports in the literature vary widely with regard to the penetration of enzymes into the wood cell wall, penetration of ligninases, Mn-dependent peroxidases, some cellulases, and hemicelluloses into the middle lamellae and/or cell wall appears to occur, at least in advanced stages of degradation.

The mechanism behind enzyme penetration into the cell wall is still unclear and it is possible that enzymes penetrate the wood cell wall only in later stages of cell wall attack. However, there is some evidence to suggest that large molecular weight glycoproteins such as degradative enzymes could move into existing microvoids in some plant tissue cell walls prior to extensive cell wall attack.

A new hypothesis for the mechanism of degradative fungal action in wood has also been proposed in this paper. The action of metal-chelating siderophores produced by decay fungi may function to chemically modify unspecified groups within wood similarly to the function of synthetic chelators and "biomimetic"' compounds reported previously by other researchers. At this point, it is unknown how closely siderophores may associate with enzymes, or if they can function in wood to penetrate into the wood cell wall, potentially "opening" the wood structure for further enzymatic attack. Research is being conducted to try to answer some of these questions.

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Decay Column Boundary Layer Formation in Maple

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INTRODUCTION

The wounding of sapwood in living trees results in a cascade of processes, including the formation of wound-initiated discoloration (Shigo, 1965). In three dimensions, this discoloration appears in the form of columns parallel to the stem axis with ragged, conical ends. All published reports indicate that in living trees, decayed wood only occurs in tissue that has undergone wound-initiated discoloration. The visible degree of discoloration and other features following wounding vary from one tree species to another. The size of wounds capable of inducing the decay process range from very small branch and root stubs to the mechanical breakage of main stems of adult trees.

Two current, conflicting concepts claim to account for the patterns of discoloration and decay initiated by wounding. The concept of compartmentalization and the alternative concept based on the maintenance of sapwood moisture content have elements in common (Shigo, 1984 cf. Boddy and Rayner, 1983). Both concepts recognize the importance to tree health of minimizing the extent of drying and subsequent death of sapwood in the vicinity of wounds. Descriptions of both concepts are acknowledged to be incomplete in matters of particular detail from one tree species to another. Both concepts claim to be parsimonious, the simplest explanation consistent with all observations and contradicted by none. The essence of the conflict is that compartmentalization invokes shifts in oxidative metabolism that result in the formation of inhibitory chemicals within distinct boundaries that serve to restrict establishment of decay fungi

and their associates. The alternative concept claims the simpler explanation that the response of sapwood to wounding is to minimize the loss of moisture. As moisture levels remain high, growth of the decay fungi is restricted without the need of chemical inhibition.

Acer saccharum L. (sugar maple) and A. rubrum L. (red maple) trees provide excellent experimental material for the study of wound responses due to the production of easily visible wound-initiated discoloration and a discrete, visibly distinct column boundary layer that separates the discolored wood from healthy sapwood (Shortle, 1979a). This research had two general objectives: (1) to determine the sequence in which the discolored wood and the column boundary layer are derived from sapwood during the initial phases of column development and to determine how wound treatments alter the initial phases of development and (2) to determine whether the process of column development is consistent with either concept that limits wound-initiated discoloration and the decay process.

MATERIALS AND METHODS

Sugar Maple Drill Wounds

Eight sugar maple trees (14 - 28 cm dbh) were wounded in June to induce column boundary layer (CBL) formation. Wounds consisted of drilling a whorl of 4 radial bore holes, evenly spaced around each stem, at 140 cm above the ground-line. Each hole was 14 mm in diameter and drilled to a depth of 50 mm.

Two trees were felled and samples collected: 1) immediately after wounding (time zero incubation), 2) during the first growing season (ca. 10 weeks following incubation), 3) at the beginning of the second growing season (52 weeks), and 4) at the beginning of the third growing season (104 weeks). Following felling, a stem bolt 20 cm in length was cut from 10 cm above to 10 cm below the whorl of drill wounds. The stem bolts were taken to the laboratory for sampling.

One drill wound from each stem bolt was selected at randomd for analysis. A 10-mm-thick (measured radially) block containing the drill hole was split from the stem bolt. This block contained wood originally located from 10 to 20 mm inward from the stem surface along the radius followed by the drill wound. Using the drill hole as a guide, the wound center-line was marked on the tangential surface of each block. A 10-mm-wide (measured longitudinally) bar was sawn from each block. The bar contained wood originally located from 5 - 15 mm above the drill hole. Each bar was split tangentially to yield sample strips that measured 5 X

10 X 60 - 70 mm (radial:longitudinal:tangential planes). Each strip was sliced at 1 mm intervals to yield samples for extraction and phenol analysis.

Each sample slice (5 X 10 X 1 mm) was placed in 5 ml of 76% ethanol and extracted at 70C for 1 hr with vortexing at 15 and 30 min. Following decanting and cooling, total soluble phenol concentrations were measured by the Folin-Ciocalteau method using one-half the standard amount of each reagent (Horowitz, 1960). Sample slices were recovered following extraction, oven-dried at 104C for 24 hr, cooled, and weighed to obtain the total mass of extracted wood. Concentrations were expressed as phenol (mg) per g of oven-dried, extracted wood. Extracts were scanned for ultraviolet (UV) absorbance over the 250 - 350 nm range.

Paraformaldehyde Treatment of Sugar Maple Drill Wounds

Two sugar maple trees (10 - 15 cm dbh) were wounded in June to determine the effect of paraformaldehyde treatment on CBL formation. Paraformaldehyde has been used to stimulate sap production from tapholes made in the course of maple sugaring. Wounds consisted of drilling a whorl of two radial bore holes in opposite faces of each stem at 140 cm above ground-line. One hole from each tree received two 250-mg paraformaldehyde pills commercially produced for use in tapholes of sugar maple. The second hole from each tree served as an untreated control. The trees were felled at 14 weeks incubation and processed as described above for sugar maple drill wounds.

Black Plastic Treatment of Scrape Wounds of Red Maple

Eight red maple trees (14 - 28 cm dbh) were wounded in June. Wounds consisted of two scrapes applied on opposite faces centered at 150 cm above ground-line. For each scrape, bark was removed in a 2.5 X 10 cm (horizontal:vertical) strip. One of the scrape wounds on each tree was covered with plastic to reduce moisture loss and impede colonization of each wound. Black plastic sheeting 0.4-mm-thick was cut slightly larger than the wound surface and held in place by two strips of plastic adhesive tape wrapped around the wound.

Two trees were felled and samples collected for phenol analysis at 4 months, 4 trees at 12 months, and 2 trees at 16 months following wounding. Sample slices were prepared as for sugar maple drill wounds, above, except that the final size of each slice was 0.5 X 5.0 X 10 mm (radial:longitudinal:tangential). Serial slices were taken along the stem radius from the center of the exposed wound surface inward to a depth of 8 mm. Each slice contained wound-initiated discolored wood, CBL, or apparently unaffected sapwood. Tissue slices were extracted and extracts analysed for phenols as described for sugar maple drill wounds. Mean

phenol concentrations for sapwood and CBL and the mean distance from the wound surface to the tissue of highest phenol concentration was determined and the least significant differences between the means were calculated ($P < 0.05$). Additional treated trees were harvested over a period of twelve years for long-term observations on CBL formation and position.

RESULTS

Sugar Maple Drill Wounds And Paraformaldehyde Treatment

Between four and six weeks were required for the formation of both the column boundary layers (CBL) and the bounded discolored wood following the application of drill wounds (Table 1). Prior to boundary formation, the mean soluble phenol concentration of sapwood located adjacent to the wound was similar to phenol concentrations of healthy sapwood located away from the wound (Table 1, Figure 1). The UV absorbance maximum of sapwood extracts was 279 $\pm$ 1 nm. Discolored wood present six weeks following wounding had a significantly lower soluble phenol concentration than contiguous healthy sapwood (Table 1). Extracts of discolored wood had a UV absorbance maximum of 281 $\pm$ 1 nm. When first formed, the CBL was greenish in color, often greenish-yellow at first and later darkening to greenish-black. Rather than a continuous sheet, the CBL appeared as a series of streaks. Eventually, the streaks formed a layer irregular in thickness and containing gaps. Later in the first year following wounding, the CBL appeared thicker and more clearly separated healthy sapwood from discolored wood. Extracts of boundary wood had significantly greater phenol concentrations than sapwood (Table 1) and a broad UV absorbance maximum of 330 $\pm$ 10 nm.

The distance from the wound center to the CBL was only slightly greater than the radius of the drill wound when measured after the first growing season and at the beginning of the second growing season after wounding (Table 2, Fig. 1). Following the second growing season, the distance from the wound center line to the layer of maximum phenol concentration in the CBL increased significantly (Table 2, Fig. 1). Treating the drill wound with paraformaldehyde also resulted in a significant increase in the distance from the wound center line to the boundary when measured 14 weeks following wounding (Table 2, Fig. 2). For all sampling times and treatments, there were no significant differences in phenol concentration for CBL, although the layers were formed in different spatial positions.

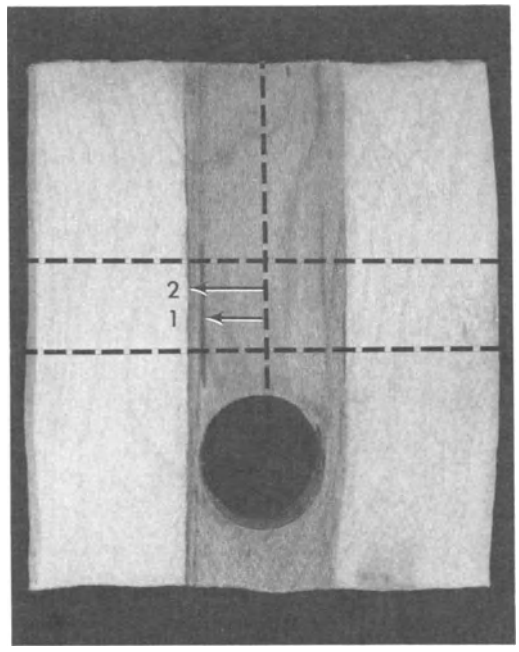

Figure 1. Drill Wound of Sugar Maple. Tangential section containing the bore hole, wound-initiated discoloration, CBL, and sapwood 104 weeks following wounding. Wood within the horizontal dotted lines was analyzed for phenols. The CBL formed during the first growing season following wounding (arrow 1) is now included within the discolored wood bounded by the CBL formed during the second growing season (arrow 2).

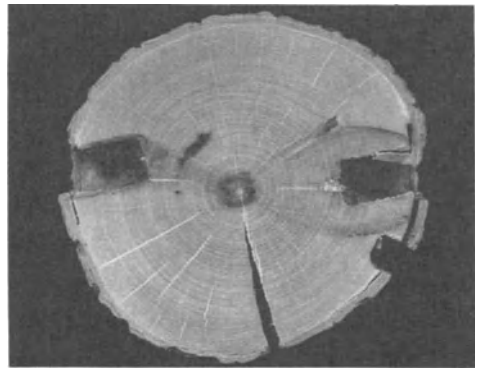

Figure 2. Drill Wounds of Sugar Maple. Transverse section showing greater amounts of wound-initiated discoloration associated with paraformaldehyde treatment (right hole) than for the untreated control (left hole).

Table 1. Phenol Concentrations of Wood Associated with Drill Wounds of Sugar Maple[a].

Incubation Period (weeks)	Wood Types			
	Sapwood (>7)[b]	Sapwood (<7)	Discolored	Boundary
0	5.2 ± 1.0	5.3 ± 0.7	-	-
2	3.8 ± 0.7	3.9 ± 0.3	-	-
4	5.1 ± 0.8	5.1 ± 0.8	-	-
6	4.1 ± 0.2	-	2.0 ± 0.3	13.3
8	5.2 ± 0.4	-	2.0 ± 0.4	7.3
18	5.5 ± 0.2	-	2.3 ± 1.6	12.7
52	5.2 ± 1.2	-	1.3 ± 0.3	14.4
104	5.2 ± 0.3	-	1.8 ± 0.7	12.2 ± 0.8

[a]Values in the body of the table are mean concentrations of soluble phenols expressed in mg/g of wood. The confidence intervals are calculated for those means composed of four or more observations ($P < 0.05$).

[b]Sapwood (>7) indicates that samples were taken more than 7 mm away from the center-line of the wound (Figure 1). Sapwood (<7) indicates that samples were taken less than 7 mm away from the center-line of the wound and consequently taken from wood that eventually would discolor.

Table 2. Distance from the Wound Center to Maximum Phenol Concentration in the Column Boundary Layer of Untreated or Paraformaldehyde Treated (PFA) Drill Wounds of Sugar Maple.

Incubation Period (weeks)	Number of Observations	Phenol Concentration (mg/g)	Mean Distance to Maximum Concentration (mm)[a]
6-18	5	11.7	7.4
52	3	14.4	7.8
104	4	12.9	8.7 *
14 + PFA	4	8.3	10.5 *

[a]Asterisk (*) indicates that the mean distance from the wound center-line to the layer of maximum phenol concentration differs significantly from the mean distance of the first growing season (7.4 mm) ($P < 0.05$).

Black Plastic Treatment of Scrape Wounds of Red Maple

In all cases, the maximum phenol concentration was significantly higher for the boundary layer than for adjacent sapwood (Table 3). Treatment of surface wounds with black plastic significantly decreased the distance from the wound surface to the area of maximum phenol concentration (Table 3). However, there were no significant differences in phenol concentration between the maxima of treated and untreated wounds. The distance from the wound surface to the innermost boundary layer increased when the wood at the wound surface was cracked or penetrated by boring insects (Table 3, Figs. 3 and 4). Twelve years following wounding, a series of boundaries was present, spatially associated with earlier formed annual rings (Fig. 3).

DISCUSSION

The results reinforce and expand central elements of the process of compartmentalization of decay in living maple trees. A distinct column

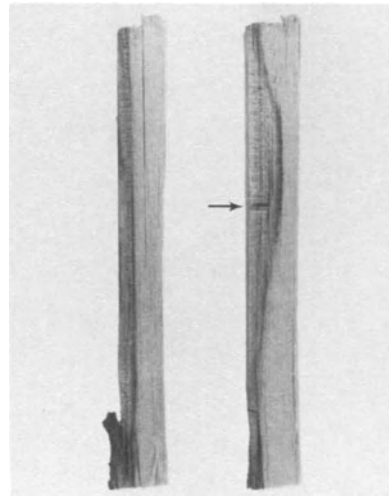

Figure 3. Scrape Wound of Red Maple. Radial section showing greater amounts of wound-initiated discoloration caused by additional wound (arrow) made by a boring insect.

Table 3. Distance from the Wound Surface to the Layer of Maximum Phenol Concentration in the Boundaries of Wounds Treated with Black Plastic and Untreated Control Wounds of Red Maple.

Incubation Period (months)	Treatment	Phenol Concentration[a] (mg/g) Sapwood	Boundary Layer	Distance to Boundary (mm)
4	Control	10	16	2
	Plastic	9	16	1
12	Control	11	23	3
	Plastic	11	33	1
16	Control	11	31	4
	Plastic	8	20	1
Overall mean	Control	11	23	3
	Plastic	10	25	1

[a]Mean of 2 (4, 16 month), 4 (12 month), and 8 (overall) observations. The least significant difference ($P < 0.05$) of the overall means were 5.3 mg/g for phenol concentration and 0.9 mm for the distance to the maximum concentration. Samples for sapwood phenol concentration were taken 5-6 mm from the wound surface.

Figure 4. Scrape Wound of Red Maple. Twelve years following wounding has resulted in partial closure of the wound. Numerous CBL are visible in transverse section and are spatially associated with cracks in the wound surface and successive growth rings.

boundary layer (CBL) separated wound-initiated discoloration from apparently unaltered sapwood. The CBL and the bounded wound-initiated discoloration were always observed together. The CBL was visibly distinct and contained greater amounts of phenol than contiguous sapwood or wood discolored by wounding (Table 1, Table 3). The shift in UV absorbance from the sapwood mean maximum of 279 nm to the CBL mean maximum of 330 nm indicates a qualitative as well as a quantitative difference in the phenols present. Compounds accumulating in the CBL appeared primarily to be derivatives of the hydroxycoumarin, fraxitin (Rowe, 1982) unlike the phenols of sapwood which are predominately derivatives of the readily oxidizable gallic acid and catechin (Tattar and Rich, 1973). This is indicative of the shift in oxidative metabolism previously described in living wood following wounding (Shortle, 1979b). The production of fungitoxic or fungistatic metabolites following wounding appears to be a common theme within the sapwood of trees (Kemp and Burden, 1986). The discolored wood within the CBL contained significantly less phenol than sapwood outside of the CBL and for sapwood not yet discolored by wounding

(Table 1). Qualitatively, the phenols present in discolored wood appeared to be derived from the phenols of sapwood by oxidative processes. Major compounds accumulating in the discolored wood are organic acid anions that account for almost one-half of the water-soluble dry matter (Shevenell and Shortle, 1986).

The biocide paraformaldehyde caused the CBL to form at a greater distance from the drill wound (Table 2, Figure 2). Apparently, the formation of the CBL requires the active participation of living cells in the sapwood. Also, carbohydrate reserves within the killed cells were not converted to host secondary metabolites, and were available for assimilation by microorganisms associated with the decay process. However, statistically similar concentrations of phenol were present in CBL associated with wounds treated with paraformaldehyde compared with nontreated control wounds (Table 2). Consequently, the biocide did not alter production of the phenolic constituent of the CBL, but did cause the CBL to be formed at a greater distance from the wound. Treatment of drill wounds with paraformaldehyde can increase the frequency of isolation of decay fungi from discolored wood (Walters and Shigo, 1978).

Column boundary layers border volumes of discolored and decayed wood induced by wounding and frequently persist for many years (Shigo, 1966). The composition of the CBL indicates that phenols may play a role in the efficacy of the CBL in limiting the spread of the decay process. The CBL is not an absolute barrier to the process of discoloration and decay following wounding. The CBL may be breached by mechanical action of increased internal dessication or hyphal penetration (Figure 1), insect boring (Figure 3), or surface cracks or checking (Figure 4). Given the continued vitality of sapwood tissue, a new CBL may be formed as a previous boundary is breached (Figures 1, 3, and 4). New boundaries are formed at the cost of energy expended during metabolic shifts, loss of functional sapwood, and increased volumes of wound-initiated discolored wood that may then later decay within the living tree. Covering scrape wounds with black plastic caused the CBL to form closer to the wound face, reducing the volume of sapwood altered by the discoloration and decay process (Table 3). Protection from dessication only partially explains the beneficial effect of the black plastic treatment. When black and clear plastic sheets were compared as wound treatments, trees with wounds treated with clear plastic had a greater frequency of occurrence of decay fungi than did trees treated with black plastic (Shortle and Shigo, 1978). This may be interpreted as the result of photo-oxidation and reduction in fungitoxicity of the phenols. Also, carbohydrate reserves within the killed cells were not converted to secondary metabolites of the host and

were available for assimilation by microorganisms associated with the decay process.

According to the alternative view to compartmentalization to account for the decay process, decay fungi and associated microorganisms grow freely through sapwood that has become reduced in moisture content following drought or through aeration associated with wounding (Boddy and Rayner, 1983). Indeed, the immediate effect of wounding sapwood is the localized loss of moisture and increased aeration (Smith, 1988). The first elements of compartmentalization include features that restrict the loss of water and maintain sapwood function, as described for the alternative view. In association with phenol enrichment, a suberized "waterproofed" zone that restricts water loss and perhaps also provides a physical barrier to hyphal penetration has been described for silver maple (Acer saccharinum L.) following wounding (Pearce and Woodward, 1986).

The alternative explanation falls short in that it does not account for the increased quantities and altered qualities of phenols in the CBL. These compounds are formed by the sapwood at metabolic expense and have been shown to inhibit the development of decay fungi in vitro and may play a role in the succession of fungal colonization of wood following wounding (Smith, Blanchard, and Shortle, 1981). Explanations for these patterns of wound-initiated discoloration ought to be parsimonious, but not made simpler than the evidence warrants.

SUMMARY

Column boundary layers (CBL) separated wound-initiated discoloration from healthy, unaltered sapwood in red and sugar maples. The formation and composition of the CBL was consistent with the concept of compartmentalization. Compartmentalization is the boundary-setting process that limits the extent of wood discoloration and subsequent decay following the wounding of living trees. The visibly distinct CBL contained greater amounts of ethanol-soluble phenol than either discolored wood or healthy sapwood. The phenols in the CBL were also qualitatively different from those in sapwood or discolored wood. Treating drill wounds with paraformaldehyde resulted in the formation of a CBL at a greater distance from the wound. This suggests that CBL formation is a physiologically distinct process that involves active shifts in secondary metabolism in response to wounding. Although these layers were always present around columns of discolored wood, a CBL was not an absolute barrier to the discoloration and decay process. As a CBL was breached due to insect damage or checking, a new CBL was formed at a greater distance from the wound, resulting in a greater volume of discolored wood. Treating scrape

wounds with black plastic resulted in the formation of CBL closer to the wound, resulting in a smaller volume of discolored wood produced by the wound. These results illustrate the dynamic, defensive nature of the tree response and the potential for cultural practices that improve or detract from the effectiveness of that response.

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Wood Decay: A Review Including Recent Developments

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INTRODUCTION

Organisms Involved in Wood Decay

Wood decay of living trees, which has been explained by the CODIT (compartmentalization of decay in trees - Shigo and Marx, 1977) model and/or the functional compartmentalization concept (Shigo, 1984), results in substantial losses of revenue to the United States each year. This decay is caused via a succession of micro-organisms (Shigo, 1967) but, in the main, by a group of wood-rotting fungi, the Hymenomycetes (Shigo and Sharon, 1968). The latter belong to the Basidiomycetes, a fungal Division in which fruiting bodies, basidiocarps, are produced. In this connection, fungi appear to be more significant than bacteria in the biodeterioration of wood (Henningsson, 1988).

Wood-destroying fungi can be classified as either white - or brown-rot fungi (Nilsson, 1979). Whereas the former degrade both cellulose (a glucan polymer) and lignin (a coniferyl alcohol polymer), the latter decompose only cellulose leaving the lignin relatively unchanged (Highley and Kirk, 1979). In addition, the white-rot fungi, unlike the brown-rots secrete polyphenol oxidase into the external milieu (Nobles, 1958; Kirk and Kelman, 1965; Taylor et al., 1987b; Moore et al., 1989). This enzyme is capable of converting o-diphenols to o-diquinones (Fric, 1976), a reaction which may be relevant to some host-pathogen interactions underlying wood decay.

Prior to its decomposition by decay fungi, wood appears to be altered by a complex interaction involving non-decay organisms, decay

fungi and the response of the host's tissue as well (Tattar et al., 1971; Shigo, 1967, 1972, 1984). These organisms are believed to cause wood decay as a succession of phenol-tolerant "pioneers" (first organisms to invade a wound, i.e., bacteria and certain fungi, yeast, blue stain fungi (Henningsson, 1988) and phenol-sensitive decayers (wood-decay fungi) acting as either pathogens or saprobes (Shortle and Cowling, 1978). The growth of these organisms seems to be modulated by postulated "limiting factors" (vitality, preservative, and solubility factors) which may operate in a temporal fashion (Shortle, 1979). The bacteria, which increase the water permeability of wood, are erosion, cavitation, and tunneling varieties (Henningsson, 1988). Bacterial attack of wood is usually observed in wood where fungal activity is reduced (Nilsson and Daniel, 1988).

Subsequent to the occurrences of decay fungi, the last stages of succession can involve not only bacteria and fungi but, in addition, protozoa, nematodes, insects, birds, and mammals. Thus, tree decay has been envisioned as a disease induced by a succession of micro-organisms (part of CODIT model) which are parasitic, saprophytic, and pathogenic (Merrill and Shigo, 1979) and influenced by other organisms.

The mentioned host response seems to yield phenolic compounds which are thought to be disease-resistance factors (Scheffer and Cowling, 1966). In this connection, Taylor et al. (1989) reported differential effects of catechol and tannic acid upon the growth of Coriolus versicolor, a white-rot fungus in culture. The biochemical mechanism(s) by which these compounds accumulate during decay most likely centers around a sapwood parenchyma cell-based shift in oxidative metabolism from the glycolytic and TCA cycle pathways to an acetate and pentose shunt-shikimic acid-pathway (Kuc, 1967; Harborne, 1980). The resultant ß-storesin and phenolic products (preservative factors) of the pathway appear to be capable of influencing certain fungal growth either negatively or positively. For example, exogenous catechol differentially affects C. versicolor's hyphal ultrastructure and growth by inhibiting the latter when added to a liquid culture medium 3 days post-inoculation but stimulating it subsequent to a 12 day supplementation (Taylor et al., 1987a, 1988). Besides this phenolic's effect, cinnamic acid (a pentose shunt shikimic acid pathway product) has been reported to suppress the growth of a decay fungus (Shortle et al., 1971). With respect to limiting factors, ß-storesin and phenolic compounds appear to act as solubility factors by "sealing-off" tissue.

COMPOSITION OF WOOD AND ENZYMES INVOLVED IN WOOD DECAY

Certain trees consist of both sapwood (immediate product of cambial derivatives) and heartwood (a dry transformation product of sapwood). The heartwood, which is thought to provide mechanical support, is wood that has been modified via genetically-controlled aging processes (Shigo, 1984). This aging promotes a change in the parenchyma cells so that they are devoid of living contents thereby preventing their functioning in storage. When present, decay fungi are confined mainly to the heartwood. In contrast, sapwood may be free of infection for years while the neighboring heartwood may be extensively destroyed.

The compartmentalization view of wood decay, advanced by Shigo (1984), was proposed to be two-componented. The first of these is the formation of a chemical reaction zone by the sapwood's living parenchyma cells as a consequence of enzymatic reactions. The production of the reaction zone is utilitarian in that it retards the spread of pathogenic micro-organisms in those tissues which were present at the time of injury.

In addition, if the sapwood is either wounded or decay-fungi encroach, the vascular cambium can respond by depositing the "barrier" zone (Buisman, 1935), a layer of cells separating the normal tissues produced prior to wounding and those formed subsequent to wounding (Tippett and Shigo, 1981). The "barrier" zone appears to be non-conducting (Mulhern et al., 1979) containing traumatic resin ducts in conifers (Tippett and Shigo, 1981).

The "barrier" zone's cells, which may be either extensive or limited in occurrence, are impervious to most wood-and-bark-localized bacteria and fungi (Shigo, 1984) thereby restricting the development of decay and discoloring fungi (Shigo, 1984). This restriction could result from alterations in the enzymatic activity of "barrier" zones as Smith et al. (personal communication) observed that peroxidase and indole-3-acetic acid (IAA) oxidase activities were higher in homogenates of wounded, red maple cambial tissue than in homogenates from non-wounded, cambial tissue. Thus, Smith et al. view their data as being consistent with an IAA-mediated formation of the "barrier" zone. Finally, some investigations have been performed relative to the chemical composition of compartmentalization barriers in oaks (Pearce and Holloway, 1984).

Another view of wood decay containing certain of the compartmentalization concepts is the previously mentioned CODIT model (Shigo and Marx, 1977). This model states that trees combat decay by strengthening "walls" of which there are four in number with three

forming part of the reaction zone. While wall 1 (a "plugging component") is the weakest and resists the vertical spread of pathogens, wall 2 ("an anatomical component") inhibits the lateral spread and wall 4 ("a differentiation component") is a tree response and a wall not in place at the time of injury. The latter wall is generated by the cambium and separates the infected wound from newly forming healthy wood. The morphology of wood following tree injury has been set forth in pictorial fashion (McGinnes et al., 1977).

Besides the formation of the aforementioned zones, wood decay, with decolorization being the first step in the decay process (Shigo and Hillis, 1973), seems to be accompanied by pertubations in the "electrical" properties of wood. Specifically, Shortle and Smith (1987) found that the "electrical" properties of aqueous extracts from various types of balsam fir were associated with differential rates of Haematostereum sanguinolentum-induced decay. Wood which was located interior to sapwood, non-dissolved and of relatively low ionization decayed slowly but identical wood possessing a high ionization decayed faster. These "electrical" properties most likely reflect fluctuations in ion content, i.e., K^{+}, Ca^{++}, acetate and formate if the data from red maple (Shevenell and Shortle, 1986) can be extrapolated to balsam fir. As for the significance of electrical phenomenon, they may be signals that initiate the formation of boundaries.

Route of Fungal Hyphae During Wood Decay

The penetration of soft rot fungal hyphae through wood during the decay process has been mapped by Hale and Eaton (1985a, b, c, 1986, 1988). They have considered the release of hyphal enzymes and excavation of cavities as mechanisms for the soft-rot-induced decay of wood.

Recently, additional progress has occurred in tracing the route of hyphal penetration through a tree's wood zones during the course of decay. This progress resulted from the development of an immunodot-blot technique/semi-quantitative detection system for Lentinus lepideus (a brown-rot fungus) and an immunocytochemical procedure for C. versicolor (a white-rot fungus) hyphae by Palfreyman et al. (1988). The antisera employed in these methods were prepared by multiple immunizations of mycelial "preparations" suspended within Freunds complete or incomplete adjuvant. However, insufficient experimental details were published by Palfreyman et al. to make a judgement regarding the purities of either the injected antigen or the resultant antibody (antisera generated). In this connection, Palfreyman et al. recognized the necessity for monoclonal antibodies which would render their immunological probes more

specific and/of value in fully discerning the cellular/tissue route by which fungal hyphae both penetrate and degrade trees of economic importance. It may be advantageous to employ fluorescein-tagged antibodies rather than the peroxidase antibody conjugate employed by Palfreyman et al. as peroxidase is widely distributed in plant tissues which could obscure the localization of hyphae detected with a peroxidase antibody conjugate. Indeed, Palfreyman et al. noted that application of a PAP - immunocytochemical method to wood sections indicated that non-specific reactions of antisera occurred with both infected and uninfected wood.

MECHANISMS OF WOOD DECAY

Involvement of Enzymes

The decomposition of wood by white-rot-fungi is accomplished by secreted cellulolytic and ligninolytic (Rosenberg, 1979) enzymes as well as peroxidase (Koenings, 1971). With regard to the latter enzyme, Reddy and Kelly (1986) presented evidence that glucose oxidase-produced H_2O_2 is involved in lignin degradation by the white-rot fungus, Phaneorchaete chysosporium. In addition to these enzymes, certain white-rot fungi produce an extracellular laccase which appears to play a role in the biotransformation of lignin (Ishihara and Miyazaki, 1972; Ander and Eriksson, 1978) and vanillic acid apparently through polymerizing syringic acid (Liu et al., 1981). In this situation, the nomenclature for phenol oxidases within the wood decay literature is confusing. Phenoloxidase appears to be a complex of o-diphenol:oxygen oxidoreductase (EC 1.10.3.1) and p-diphenol:oxygen oxidoreductase (EC 1.10.3.2). Whereas the former is known as polyphenol oxidase, tyrosinase, or DOPA-oxidase converting o-diphenols to o-diquinones, the latter is known as laccase and catalyzes the conversion of p-hydroquinones or p-phenylenediamines to p-quinonediimines (Fric, 1976). Extracellular laccases from decay fungi have been investigated by Bollag and Leonowicz (1984). In addition, some fungi contain m-dihydroxyphenolase which catalyzes the aerobic oxidation of dihydroxyphenols with the polyphenol oxidase of wood-rotting fungi being involved in lignin degradation. The time-dependent appearance of both intracellular and extracellular C. versicolor polyphenol oxidase has been described by Moore et al. (1989).

Besides the white-rot fungi, the brown-rot fungi can also secrete extracellular cellulases (Greaves, 1971). Thus, wood decay is not limited to the white-rot fungi as already stated.

One of the ligninolytic enzymes which has been the subject of recent considerable research interest is ligninase. This enzyme has been both

isolated and partially characterized from P. chrysosporium (Tien and Kirk, 1984). This hemoprotein enzyme catalyzes the H_2O_2 - dependent oxidation of 3, 4-dimethoxybenzyl (veratryl) alcohol yielding veratraldehyde. Furthermore, it appears to exhibit a peroxidase mechanism for its action on lignin-like substrates. The production of ligninase by P. chrysosporium has been examined by Kirk et al. (1985), Jager et al. (1985), Leisola et al. (1985), and Kirkpatrick and Palmer (1987).

Although most studies on wood-decay have concentrated on the degradation of lignocellulose in terrestrial environments, there are some investigations concerned with the biodegradation of lignocellulose in aquatic environments (Hodson and Benner, 1986; Benner et al, 1986). These results raise the potential of commercial applications of anaerobic lignin transformations concerned with the biodegradation of lignocellulose in aquatic environments (Hodson and Benner, 1986; Benner et al., 1986).

Role of Free Radicals

An area of wood decay research which has received considerable emphasis of late is that involving the location, source and function of free radicals. The free radical contents of wood have been identified and their decay behavior investigated by Ou et al. (1984). In addition, Jekel et al. (1981) examined the free radicals in spruce wood in relation to white-rot induced damage and Illman et al. (1989) have detected the oxygen free radical in wood colonized by the brown-rot fungus, Pastia placento. The latter contend that their data support the hypothesis that brown-rot fungi decompose wood by an oxygen-free-radical mechanism. As for the mechanisms by which free radicals are generated, they appear to originate by both photo-irradiation at ambient temperature (Hon and Frist, 1981) and gamma irradiation at -196°C (Belkova et al., 1985). The source of at least certain of the free radicals seems to be the photolysis of lignin (Ranby, 1969; Hon and Frist, 1981; Hon, 1981; Belkova et al., 1985) with water molecules facilitating light penetration into wood thereby possibly enhancing the photolysis of lignin (Chupka and Rykova, 1980).

FUTURE PROJECTIONS - CONTROL OF WOOD DECAY

Employment of Regulators of Fungal Growth

Although efforts continue to develop effective wood preservatives, more attention should be given to investigating tree-derived phenolic compounds as possible regulators of fungal growth. For example, the

recent results of Taylor et al. (1987 a, b) indicate that C. versicolor's growth in liquid culture can be affected in bimodal fashion via the time-dependent application of catechol, a naturally occurring phenolic compound. In addition, the employment of inhibitors (e.g., diethylthiocarbamate) of fungal polyphenol oxidase (Taylor et al., 1987a) may warrant testing as fungal growth control agents in situ.

The possibility that phenolics may regulate hyphal growth in a bimodal fashion through the products of extracellular polyphenol oxidase activity was raised by Taylor et al. (1987b) who provided data which supported the contention within the literature that the products are quinones (Fric, 1976). What was both new and fascinating was that these purported quinones appeared within a liquid growth medium in a highly time-dependent manner corresponding to a marked catechol-induced stimulation of mycelial growth as well as pertubations in subcellular morphology (Taylor et al., 1988). These observations prompt a need for a thorough examination of the efficacy of quinones as fungal growth control agents.

Another area of research which should be afforded considerable attention involves the toxicity of biocides toward fungi involved in wood decay (Smith and Ingleby, 19B5). This is being examined by Williams and Eaton (1988). With regard to wood preservation, Butcher (1988) pointed out that attention should be focused on three areas of research, i.e., application of systems for antisorption chemicals, "just-in-time" processing (immediacy in preservative treatment) and the use of boron-based wood preservative systems.

Use of Biological Control Mechanisms

This seems plausible as Smith et al. (1981) presented evidence that Trichoderma harzianum can be employed as a biological control agent against wood-decay fungi such as Fomes connatus, a causative organism of red maple tree decay. The mechanism by which T. harzianum accomplishes this has been hypothesized to involve a replacement of "pioneer fungi", a hypothesis worth investigating.

Genetic engineering - Instead of conventional breeding, the employment of genetic engineering techniques (Maniatis et al., 1982; Wu, 1983) may be the answer to the rapid generation of trees resistant to decay organisms. For example, DNA fragments carrying the genetic information for resistance could be excised from the genome of resistant trees by restriction endonucleases (Maniatis et al., 1982), characterized (Williams, 1985) and subsequently incorporated into vectors for the infection of sensitive trees. The ultimate goal of these manipulations would be the insertion of DNA fragments bearing the information for

resistance into the sensitive tree's genome (Williams et al., 1981; Baylor et al., 1983). This approach is appealing for it would eliminate the need for costly wood preservatives whose toxicity and long-term pharmacological effects to animals and man are not always known.

Finally, since it is not possible to adequately review the extensive literature regarding the diverse aspects of wood-decay, the reader is referred to the comprehensive bibliography of Shortle (1984), the monograph of Higuchi (1985) and the reviews of Smith (1989) as well as Illman and Highley (1989) to gain an indepth view of both past and current views of wood-decay.

SUMMARY

The evidence that wood decay is a disease which can be explained by CODIT (compartmentalization of decay in trees) model and/or the functional compartmentalizaton is reviewed. In addition, the organism and enzymes involved in wood decay are discussed. The route of fungal hyphae as well as the mechanism of wood decay including the possible role of free radicals in the decay process are emphasized. Finally, the direction in which wood decay research may proceed are offered.

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Acknowledgments

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Coriolus versicolor, A Model System to Investigate the Biotechnology of Wood-Deteriorating Enzymes

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INTRODUCTION

Coriolus versicolor, a white-rot basidiomycete, elaborates cellulolytic and ligninolytic enzymes both in situ and in vitro (Evans and Palmer, 1983; Taylor et al., 1987, 1988; Moore et al., 1989). These enzymes can degrade cellulose (Peterson et al., 1963) and lignin (Harvey et al., 1987), the main polymers of wood (Higuchi, 1985). Besides its presence within wood, lignocellulose often constitutes an unwanted component in the paper-pulp industry and also renders certain agricultural commodities less digestible to ruminants (Dodson et al., 1986; Van der Meer et al., 1987). Thus, an available supply of ligno-cellulolytic enzymes could be of marked industrial value. To maintain an adequate supply, substrate induction coupled to hyphal 'batch culture' (Fahraeus and Reinhammer, 1967) and/or genetic engineering techniques (Maniatis et al., 1982; Bennett and Lasure, 1985; Current Protocols in Molecular Biology, 1987) are being sought to over-produce these enzymes. Here are reported pertinent recombinant DNA techniques (Williams et al., 1988, 1989a,b) which may result in enhanced enzyme synthesis by Escherichia coli transformants harboring the C. versicolor gene(s) for polyphenol oxidase (PPO), an enzyme relevant to lignin degradation (Kirk and Kelman, 1965) as well as converting the purported disease-resistance, phenolic compounds (Taylor et al., 1989) to diquinones (Fric, 1976).

MATERIAL AND METHODS

Growth of Coriolus versicolor

Coriolus versicolor (L. ex fr.) Quel [isolate from the USDA-Northeastern Forest Experiment Station (Durham, NH)] can be cultured

according to Taylor et al. (1988) and Moore et al. (1989) in a defined liquid medium (Kirk and Kelman, 1965).

Isolation of Genomic DNA

Two procedures can be utilized to successfully isolate Coriolus versicolor hyphal genomic DNA. These are the 'bacterial method' of Maniatis et al. (1982) and the plant procedure listed in Current Protocols in Molecular Biology (1987). These procedures are summarized in flow chart form within Figure 1.

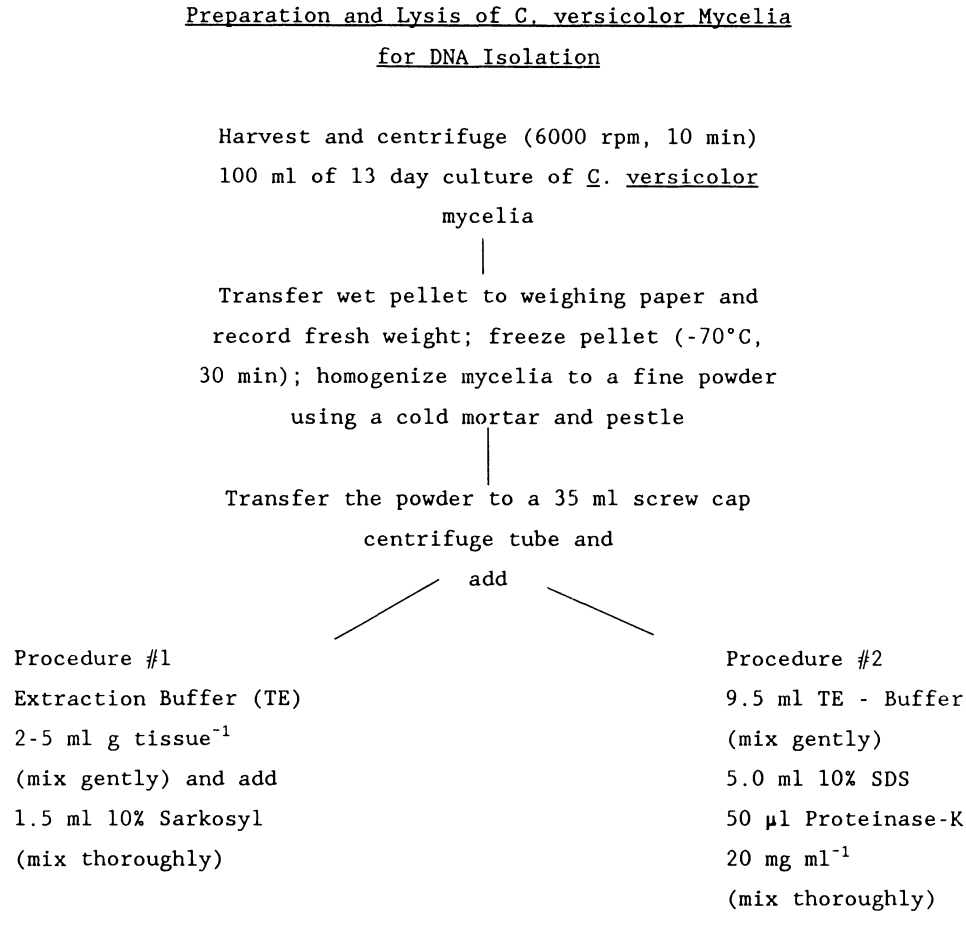

Figure 1. Summary of Procedures for the Isolation of Coriolus versicolor Genomic DNA Bacterial Procedure (1) and Plant Method (2).

Precipitation of DNA

Centrifuge (6000 rpm[a], 10 min); collect supernatant

↓

Add 0.6 volume isopropanol (mix gently); observe nucleic acid precipitate

↓

Collect precipitate (pellet) via centrifugation (8000 rpm, 15 min, 4°C)

↓

Resuspend pellet in 9 ml TE-Buffer and add 9.7 g solid $CsCl_2$ (mix)

↓

Incubate on ice 30 min

↓

Centrifuge (8000 rpm, 10 min, 4°C) and retain supernatant

↓

Add 0.5 ml of ethidium bromide (10 mg ml^{-1})

↓

Incubate on ice 30 min

↓

Centrifuge (8000 rpm, 10 min); collect supernatant

↓

Add 1.8 ml of 5.0 M NaCl; mix and add 1.5 ml CTAB/NaCl (mix thoroughly)

↓

Incubate 20 min, 65°C

↓

Add an equal volume of chloroform/isoamyl alcohol (24:1) extract thoroughly

↓

Centrifuge at room temperature (6000 rpm) to separate phase

↓

Collect the aqueous phase; add 0.6 volume of isopropanol; observe precipitation (stringy white DNA mass)

↓

Collect DNA using a hooked pipet and transfer to a centrifuge tube; add 1 ml of 70% alcohol

↓

Centrifuge (9000 rpm, 5 min) obtain pellet; resuspend in 8 ml TE-Buffer

↓

Quantitate DNA at A_{260nm} with a spectrophotometer; adjust conc. to 100-200 µg ml^{-1}; add 8.6 g $CsCl_2$ 8 mls of DNA-TE suspension^{-1}

↓

Add 40 µg ethidium bromide (10 mg ml^{-1}), mix gently

(Continued)

Transfer to Quick seal centrifuge tube (13.5 ml); balance

↓

Centrifuge overnight (55,000 rpm[b], 15°C)

↓

Visualize gradient under longwave UV lamp; collect DNA band with 18 G-needle

↓

Remove ethidium bromide by sequential extractions with H_2O-saturated butanol

↓

Dialyze overnight against TE-buffer (2 times)

↓

Concentrate by adding 1/10 volume NaOAC and 2 volume 100% alcohol; allow to ppt. overnight 20°C or 1-2 hr - 70°C

↓

Centrifuge (10 min, 10,000 rpm); collect pellet

↓

Wash 2X with 70 alcohol

↓

Dry pellet (speed vac.; 30 min)

↓

Resuspend in 100 - 500 µl TE-Buffer

↓

Measure concentration and store 0.5-1.0 µg μl^{-1}

↓

Agarose gel analysis; add (200 ng $well^{-1}$) for confirmation of isolated DNA

---

[a]6,000, 8000, 9,000 and 10,000 rpms can be generated with a JA20 rotor in a Beckman J-21 centrifuge

[b]55,000 rpms can be obtained with a Beckman type 65 rotor in a Beckman L8-70 M ultracentrifuge

Fig. 1 (continued)

Treatment of Isolated Genomic DNA with Restriction Endonucleases

To derive a C. versicolor DNA fragment which possesses the gene(s) for PPO, isolated and purified genomic DNA was treated with restriction endonucleases under appropriate conditions. Table 1 presents the restriction endonucleases which were employed to digest genomic DNA in order to generate fragments which can be ligated into vectors for use in Escherichia coli transformations.

Table 1. Restriction Endonucleases Used Against C. versicolor DNA[a]

Enzyme	Buffer[b]	Recognition Sequence	Compatible Cohesive Ends
BamHI	medium	G↓GATCC	BglI, BglII, MboI, Sau3A, XhoII
EcoRI	low	G↓AATTC	EcoRI
BglII	low	A↓GATCT	BamHI, BglI, MboI, Sau3A, XhoII
HindIII	medium	A↓AGCTT	----------

[a]A typical restriction enzyme reaction involves DNA (2-4 μg), 10X restriction buffer (95 μl), restriction enzyme (5-10 U μg^{-1}) and sterile distilled water in a final reaction volume of 50 μl. The reaction is incubated for 1-3 h at 37°C. Subsequent to the incubation, the sample is heated for 5 min at 65°C. Then aliquots are removed and treated with 5X stop mix prior to loading onto 1.0-1.5% agarose for electrophoresis.

[b]Low buffer = 10 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$ and 1 mM dithiothreitol. Medium buffer = 50 mM NaCl, 10 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$ and 1 mM dithiothreitol.

Ligation of Restriction Endonuclease - Generated Fragments

To transform E. coli with recombinant plasmid, the fragments were employed in the ligation technique (Figure 2). With this method, a fragment of genomic DNA resulting from endonuclease digestion was inserted into a specific site of the plasmid's DNA. Then, the insertion of the fragment was verified by agarose gel electrophoresis (Maniatis et al., 1982); an example of which is depicted in Figure 3.

Transformation of Escherichia coli Cells With Recombinant Plasmid

The procedures which can be employed for the transformation of E. coli cells with recombinant plasmid are displayed in Figure 4. Of critical importance is the end-product of the protocol, i.e., can transformed E. coli cells express the gene(s) for C. versicolor's PPO (Worthington, 1982)? This involves the quantification of PPO spc. act. by enzyme assay (Evans and Palmer, 1983).

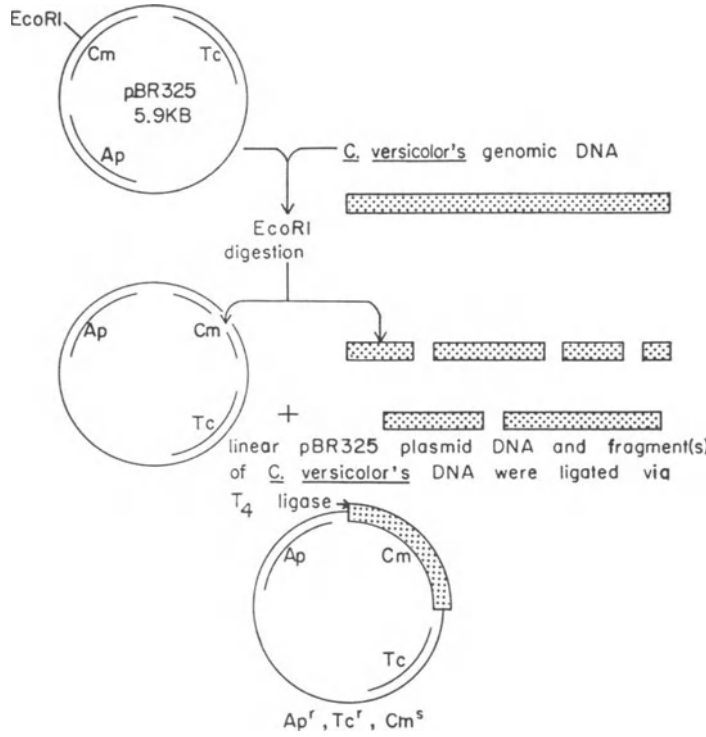

Figure 2. Generalized Cloning Scheme for Coriolus versicolor's Polyphenol Oxidase (PPO) Gene(s).

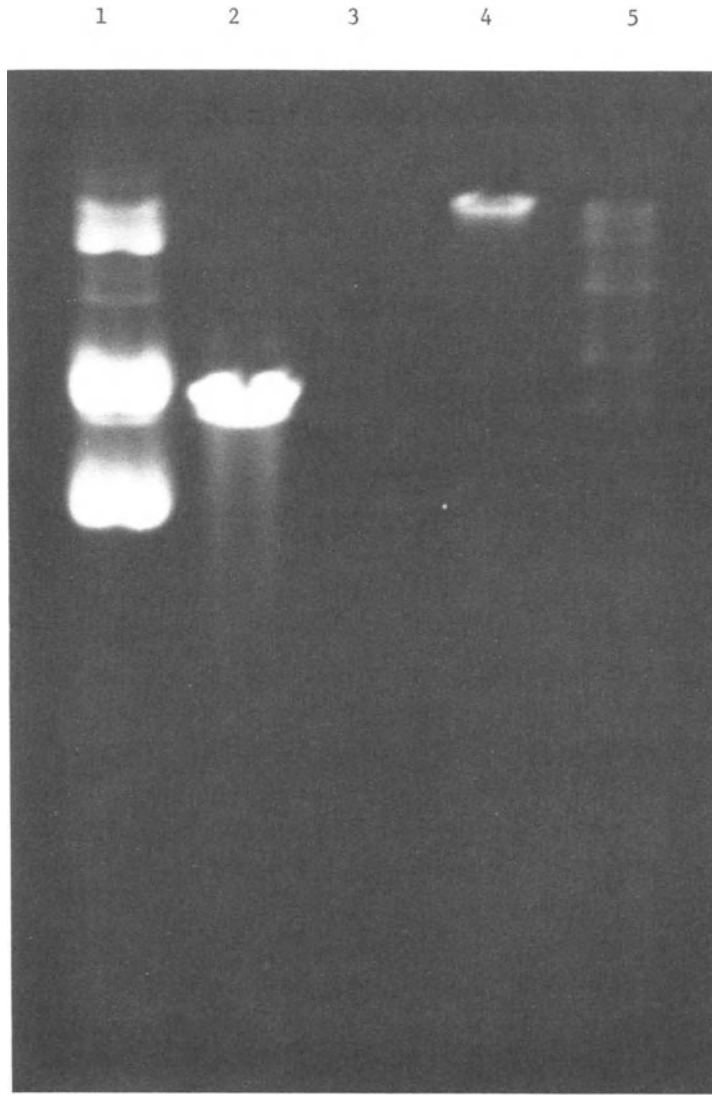

Figure 3. 1.0% Agarose Gel Analysis of Ligated Recombinant Plasmid (Confirmation of Ligation). An aliquot (200 mg DNA) of each sample was applied to agarose (1%) gel and electrophoresed at 50 V for 2-4 h. Subsequent to electrophoresis, the gel was stained with ethidium bromide (0.5 $\mu g\ ml^{-1}$), illuminated with UV light and photographed. Lanes = 1 (Intact pBR325); 2 (pBR325/EcoRI); 3 (C. versicolor); 4 (Intact C. versicolor DNA); 5 (Ligated product of 2 and 3).

Transformation via Calcium Chloride/Heat Treated Cells

I. Preparation of Competent Cells:

Grow 5 ml culture overnight

↓

Dilute overnight culture 1:50 with fresh broth
(i.e., 1 ml overnight culture + 49 ml LB)
10 g tryptone broth, 5 g yeast extract,
5 g NaCl 1 g glucose liter d.H_2O^{-1}

↓

Allow growth to early log phase
(O.D. 0.4 - 0.5) OR (1.5 X 10^8 cells ml^{-1})

↓

Remove 25 ml aliquots of cells into
35 ml screw cap centrifuge tubes

↓

Chill on ice for 10 min
(NOTE: Maintain cells upon ice
whenever they are not being centrifuged)

↓

Pellet cells at 4,000 rpm (4K), 5 min, 4°C
Discard supernatant and place pellet on ice

↓

Resuspend pellet in 2.0 ml of cold 0.1 M $CaCl_2$,
by gentle swirling
(DO NOT shake or vortex)

↓

Add 18.0 ml of additional cold 0.1 M $CaCl_2$,
swirl again, and place upon ice for 20 min

↓

Pellet the $CaCl_2$-treated cells at 4,000 rpm (4K),
5 min, 4°C
Discard supernatant and resuspend in
0.5 - 1.0 ml of cold 0.1 M $CaCl_2$;
swirl gently to resuspend
"Competent" cells
(store on ice until needed)

NOTE: "Competent cells" remain viable 18 - 24 hr

Figure 4. Flow chart of steps involved in a generalized scheme for bacterial transformation

II. Transformation

Label 3 - Eppendorf tubes: N, P, & E

N = negative control --- 100 µl "competent" cells only

P = positive control --- 100 µl "competent cells"
+ 10 - 100 ng DNA (known)

E = experimental --- 100 µl "competent cells"
+ 10 - 100 ng Test DNA
(recombinant molecule)

"Flick" the tubes to mix DNA and cells together

|

Place on ice for 15 min (undisturbed)

|

Transfer tubes to 37°C water bath for 7 min

|

Remove, add 1.0 ml of L-Broth to each tube and
place in water bath shaker at 37°C 15 - 60 min

|

Set up pre-prepared agar plates
(i.e., L-agar + - drug being selected for)

|

Plate cells as follows:

Tubes	-drug plate	+drug plate
N control	1 x 10^7 cells	0.1 ml (undiluted)
P control	1 x 10^7 cells	0.1 ml (undiluted)
E experimental	1 x 10^7 cells	0.1 ml (undiluted)

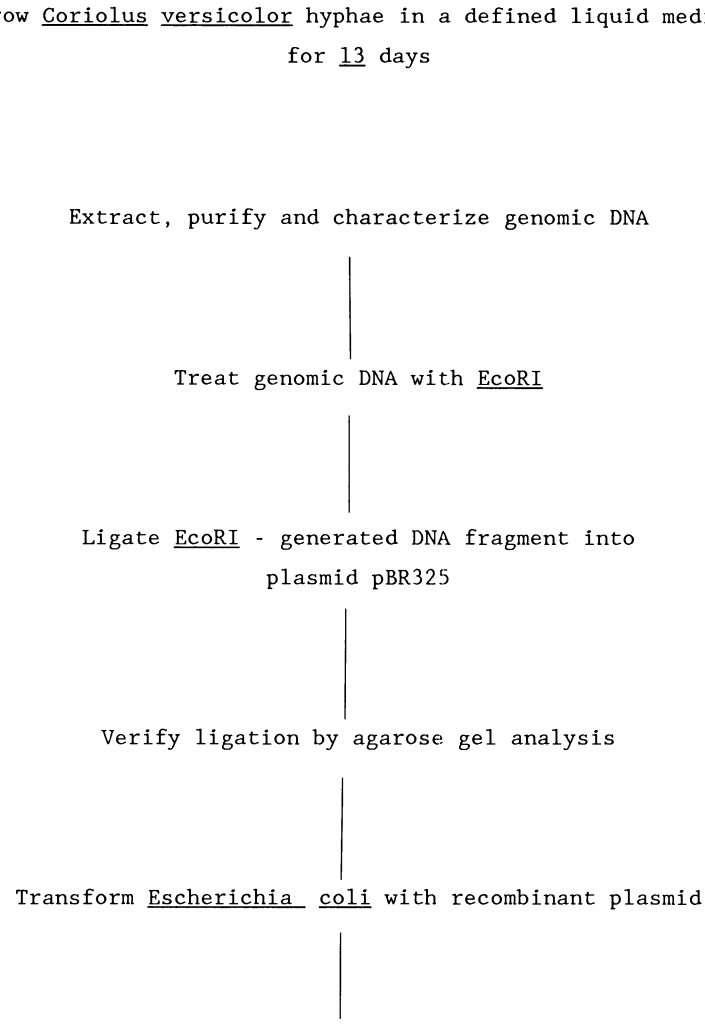

Figure 5. Flow Chart Summarizing the Molecular Cloning Techniques Utilized for Coriolus versicolor's PPO Gene(s).

Finally, the procedures which can be utilized to clone the gene(s) for C. versicolor's PPO are summarized in Figure 5.

SUMMARY

Coriolus versicolor is a white-rot basidiomycete which elaborates cellulolytic and ligninolytic enzymes both in situ and in vitro. Because the fungus can be "batch-cultured" in a defined liquid medium, it may be a "model system" to achieve over-production and enhanced secretion of wood-degrading enzymes. Here are reported recombinant DNA technologies which over-produce these enzymes. The technologies include: extraction, quantification and purification of hyphal genomic DNA, restriction endonuclease digestion of the DNA, ligation of certain EcoRI-generated fragments into plasmid pBR325 DNA and transformation of Escherichia coli with the recombinant plasmid. Next, the E. coli transformants carrying non-mutagenized and mutagenized inserts can be screened for their abilities to express the incorporated C. versicolor genes for cellulolytic and ligninolytic enzymes. Then, the genetically-engineered, wood-degrading enzymes may be employed to remove "unwanted" lignocellulosic substances for the paper-pulp industry and to render certain agricultural commodites, e.g., straw, more digestible to ruminants.

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Ovule Culture to Study Reproductive Development at the Cellular Level in Loblolly Pine (*Pinus taeda* L.)

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INTRODUCTION

Loblolly pine is an important commercial timber and pulpwood tree of the Southeastern United States (Guiness, 1982). Recently, inadequate regeneration of this pine was identified as a problem causing reduction in the volume of softwood in the Southeast. To supply adequate seedlings for regeneration, nurseries (Davey, 1982) and clonal seed orchards (Zobel et al., 1958) have been established. Successful plantations of loblolly pine can be established from nursery-grown seedlings of improved genetic strains (Dierauf, 1982). To fully understand seed development in loblolly pine (Figure 1), the culture of excised ovules could be used to study in vitro fertilization. If excised loblolly pine ovules can be maintained in culture, the production of plantlets could perhaps be enhanced by methods similar to existing procedures (Mott and Anderson, 1981).

MATERIALS AND METHODS

Conelet Collection and Excision of Ovules

Pinus taeda conelets were collected monthly from January, 1985 to June, 1986 from a Georgia Forestry Commission Seed Orchard (Pulaski County, GA). Following collection, the conelets were packed in ice and returned to the laboratory where ovules were excised.

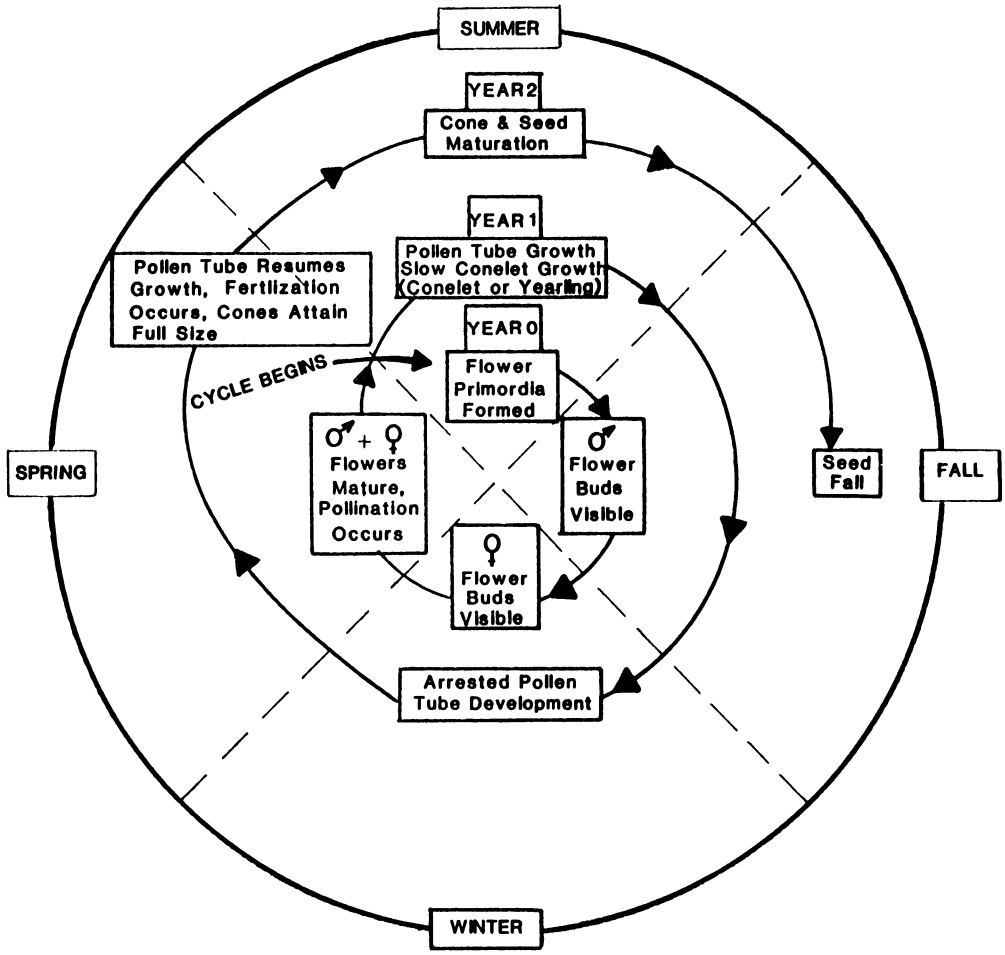

Figure 1. Life Cycle of Pinus taeda L.

Culture of Ovules

Conelets were surface-sterilized with 10% chlorox (20 min.) and subsequently washed three times with sterile distilled water. Ovules, with attached pine scales, were then excised and after a final rinse with sterile distilled water placed (in replicate groups of 8) onto an agar-solidified medium designed for the culture of loblolly pine tissues (Mehra-Palta et al., 1978). For most cultures, the medium contained 1 μM IAA, GA_3, or kinetin. Ovules were cultured at 25 ± 2°C for either one or more months. The photoperiod during culturing was 16 hr light/8 hr dark.

Histochemistry-Histochemical Preparation

Both freshly-excised and cultured ovules were fixed in Carnoy's solution (ethanol: acetic acid, 3:1) for 4.5 hr. Following fixation, the

ovules were dehydrated through a graded alcohol series and then progressively embedded in xylene-paraplast mixtures. Embedded ovules were sectioned at 10 μm with a rotary microtome.

Histochemical Localization

To localize proteins, mounted sections were deparaffinized and stained with ninhydrin-Schiff (Yassuma and Ichikawa, 1952). Azure B (Flax and Himes, 1952) was used to localize RNA and the Feulgen reaction (Jensen, 1962) was employed to detect DNA. Stained sections were subjected to microspectrophotometry to quantify stain intensities. A Zeiss microspectrophotometer (Model No. UMSP 50) was used. The instrument was programmed to yield a wavescan absorption spectrum from 500-700 nm for sections stained for protein, DNA and RNA localization and/or quantification. The spectra were double-apertured; i.e., both a measuring spot of known size (6.25 μm) and a field stop (to illuminate just the area of interest and therefore to block either stray light or glare) were used. The spectra were also corrected for any deviations which might arise from glassware and sample mounting, i.e., a spectrum of the blank slide and mounting medium were divided into the object spectrum. Stained slides were positioned in such a way that the measuring spot fell on the area of interest (nuclei for DNA and protein: cytoplasm for RNA). All figures were magnified 705X.

Measures of Ovule Viability - Respiratory Measurement

To assess the capacities of ovules to respire, a Gilson (Model No. IG-20) Respirometer was employed. The water bath which held the reaction flasks was maintained at 25°C. Each of these 15 ml flasks contained 2 ml of liquid culture medium and 50 μl aliquots of 3N KOH were added to the flasks' center wells. Then, a small piece of fluted filter paper was placed into the center well followed by greasing the side arms. Ovules in groups of 20 were placed into the liquid media within the flasks, which were then mounted onto the respirometer. The flasks were then immersed into the water and allowed to equilibrate for 10 min. after shaking. The valves were closed and μl O_2 consumed readings taken every 20 min. For the controls, ovules were not added to the flasks. Each assay was done in triplicate.

Statistical Analyses

The data were analyzed by either a t-test for comparing two population means ($N = < 30$) or an analysis of variance (Snedecor and Cochran, 1979).

RESULTS

Comparison of Respiratory Measurements of Freshly-excised Ovules and Ovules Cultured for Various Time Periods

The respiratory activities of both freshly-excised and cultured pine ovules were quantified by a respirometer and the results are depicted in Figure 2. In these assays, freshly-excised ovules obtained from cones collected at different times were compared for their respiratory activities. The comparisons also consisted of a search for either similarities or differences in respiratory activities between freshly-excised ovules and those cultured for different periods of time.

Comparison of the respiratory activities of ovules cultured for either one month (April, 1986 collection) or six months (October, 1985 collection) revealed that the rate of respiration ($\mu l\ O_2$ consumed 120 min^{-1}) for the April freshly-excised ovules was significantly higher ($p = 0.05$) than for those cultured for either one or six months. However, the difference in rate of respiration between the freshly-excised and one-month-cultured ovules was only 18%.

Comparisons of Staining Intensities of Combinations of Freshly-Excised and Cultured Ovules

The microspectrophotometer was programmed to record absorption spectra over 500-700 nm in order to obtain absorption maxima for protein, RNA and DNA. The latter two were from azure B-stained sections. Whereas DNA possessed an absorption maximum at 595 nm, RNA and protein exhibited maxima at 600 nm, respectively. An example of these spectra is seen in Figure 3A-B which depicts the spectra for RNA. Table 1 presents both means and standard deviations of absorbances for both stained cultured and freshly-excised ovules. For ovules both collected and cultured in August, the ovules cultured upon a medium to which 1 μM IAA was added and stained with azure B displayed a significantly higher absorbance when compared to the medium without hormone. Those sections from ovules cultured in either 1 μM kinetin or GA_3 and stained with azure B did not possess higher absorbances than those sections obtained from ovules cultured without hormone.

Comparisons of Cellular Dimensions and Cell Counts-Cell Dimensions

Both cell lengths and widths of freshly-excised and cultured ovules are presented in Table 2. Whereas the top portion of the Table compares cultured ovules with and without hormones, the bottom portion compares freshly-excised ovules and cultured ovules with hormones.

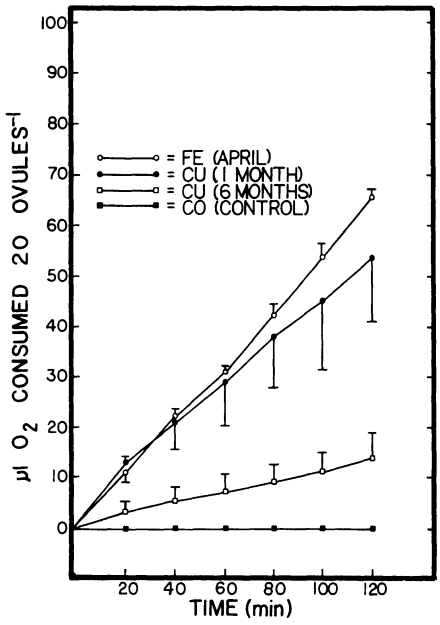

Figure 2. Respiration of Freshly-excised Ovules and Ovules Which Were Cultured for Either One or Six Months. Data are means and standard deviations where N = 3 replicate flasks each containing 20 ovules for each ovule condition.

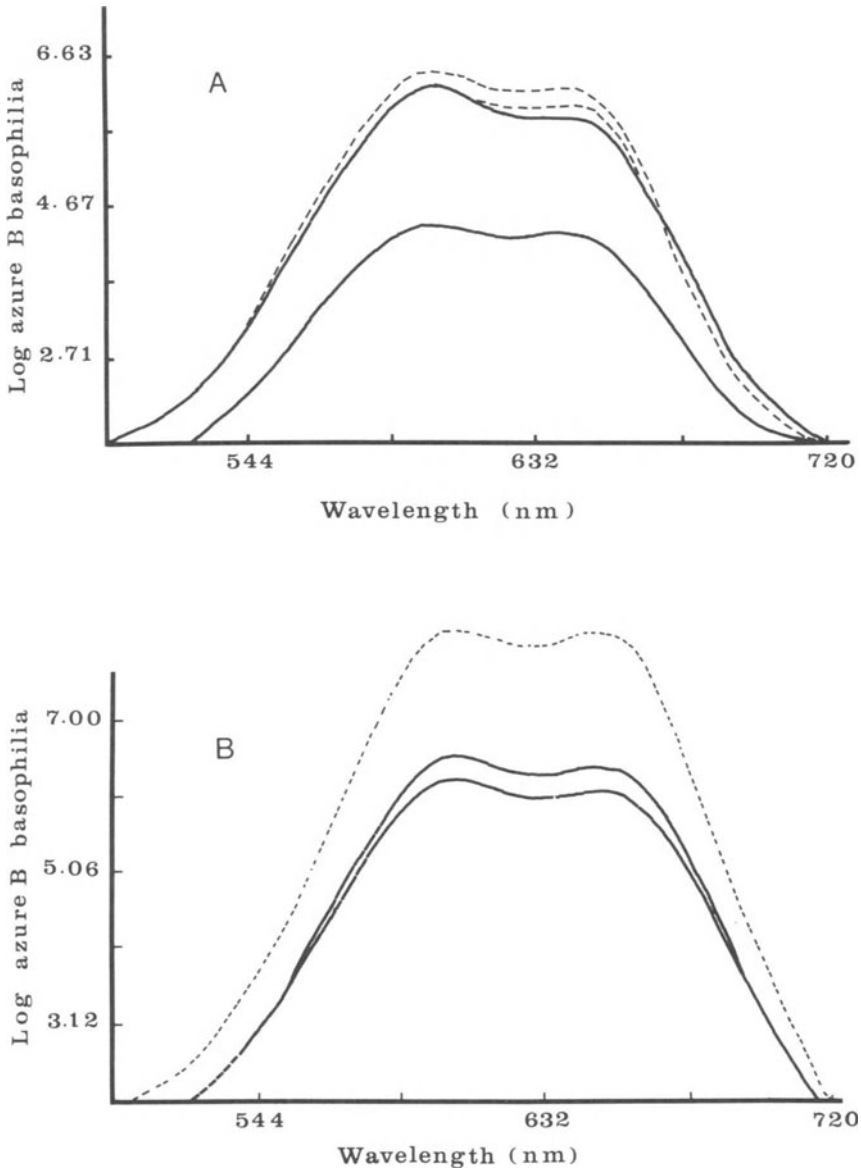

Figure 3A, B. Absorption Spectra of Azure B-stained Sections of Freshly-excised Ovules (A) and Those Cultured with 1 μM Kinetin (B). A = conelets collected in February, 1986, B = conelets collected in January, 1986. Log on Y axis = log of microspectrophotometric quantification of azure B basophilia. Various curves represent measurements for randomly selected cells from different ovules of the same collection.

Table 1. Comparison of Microspectrophotometric Absorbances Between Stained Sections from Freshly-Excised and Cultured Ovules.

Ovule Condition	Log Microspectrometrically Quantified Azure B Basophilia 6.25 μm^{-1}
Cultured versus Cultured[a]	
Without hormone	2.45 ± 0.30
With 1 μM kinetin	1.75 ± 0.40
With 1 μM IAA	3.97 ± 0.30*
With 1 μM GA_3	1.60 ± 0.50
Freshly-excised versus Cultured	
Freshly-excised[b]	6.00 ± 0.13
Cultured with 1 μM kinetin[c]	6.50 ± 0.94
Freshly-excised[b]	7.62 ± 0.05
Cultured[d]	8.04 ± 0.24
Freshly-excised versus Freshly-excised	
Freshly-excised[e]	6.95 ± 0.46
Freshly-excised[f]	7.40 ± 0.24

[a]August (1985) ovules cultured one month; sections stained with azure B for RNA.
[b]February (1986) ovules fresly-excised and fixed; sections stained with azure B or ninhydrin for total protein.
[c]January (1985) ovules cultured one month; sections stained with azure B.
[d]February (1986) ovules cultured one month; sections stained with ninhydrin.
[e]October (1985) ovules freshly-excised and fixed; sections stained with Feulgen for DNA.
[f]March (1986) ovules freshly-excised and fixed; sections stained with Feulgen.
*Indicates significantly higher value as compared to without hormone ($p = 0.05$) where $N = 3$.

Cultured versus cultured - Whereas the addition of 1 μM kinetin to the culture medium resulted in a 7.2% reduction in ovule cell lengths, supplementation with 1 μM IAA and 1 μM GA_3 yielded 1.11 and 1.12-fold increases, respectively. Of these changes, those for both IAA and GA_3 were statistically significant. As for cell widths, the addition of 1 μM kinetin, IAA or GA_3 enhanced ovule widths by 1.10, 1.32 and 1.31 folds, respectively. The enhancements were statistically significant at $p = 0.05$.

Freshly-excised versus cultured - When ovular lengths and widths for freshly-excised ovules (October, 1985 collection) and those cultured with 1 μM kinetin (September, 1985 collection) were compared, 1.38 and 1.08-fold increases in cell lengths and widths were observed for cultured ovules, respectively. Both of these elevations were statistically significant.

Except for the enhanced cell widths of the ovules cultured with 1 μM GA_3, freshly-excised (February, 1986 collection) ovules and ovules cultured with and without 1 μM kinetin (January, 1986 collection) and 1 μM GA_3 (January, 1986 collection) exhibited similar cell lengths and widths.

Finally, when freshly-excised (March, 1986 collection) ovules and ovules cultured with kinetin were compared, a 12.9% reduction in cell length but a 1.07-fold increase in width were noted.

Dimensions of ovules maintained in culture for 3 months were measured. In one case a callus was obtained. When the ovules were placed into culture, the average cell lengths and widths were 2.5 and 3.0 mm, respectively. After 3 months, these measurements were repeated and the callus cells were found to be 4 and 5 mm in their lengths and widths, respectively. The rest of the ovules were cultured with an attached pine scale.

Cell counts

A comparison of cell numbers within cytological sections of both freshly-excised and cultured ovules is shown within Table 3. It is apparent that there were no statistically significant differences in cell numbers between ovules which were cultured without hormones and those cultured with 1 μM kinetin, 1 μM IAA or 1 μM GA_3. In addition, significant differences in cell number between freshly-excised and ovules cultured with either kinetin or GA_3 were not observed.

DISCUSSION

The use of manometry to evaluate the respiration of ovules

Table 2. Lengths and Widths of Cells from Cultured and /or Freshly-Excised Ovules.

Ovule Condition	Length (μm)	Width (μm)
Cultured versus Cultured[a]		
With hormone	273.0 ± 41.1	261.3 ± 45.2
With 1 μM kinetin	253.5 ± 55.9	288.6 ± 73.9*
With 1 μM IAA	304.2 ± 77.5*	347.1 ± 74.6*
With 1 μm GA_3	308.1 ± 69.8*	343.2 ± 110.0*
Freshly-excised versus Cultured		
Freshly-excised[b]	249.6 ± 61.5	241.8 ± 44.2
Cultured with 1 μM kinetin[c]	343.2 ± 40.2*	261.3 ± 52.2*
Freshly-excised[d]	245.7 ± 26.3*	280.9 ± 54.7
Cultured with 1 μM kinetin[e]	230.0 ± 31.1	276.9 ± 38.8
Cultured with 1 μM GA_3[e]	257.4 ± 52.6	413.4 ± 64.2**
Freshly-excised[f]	304.2 ± 54.5*	269.1 ± 59.4
Cultured with 1 μM kinetin[g]	265.2 ± 54.5	288.6 ± 64.2**

Data are means and standard deviations where N = 10.
*Statistically different from cultured with or without hormone at $p = 0.05$.
**Statistically different from freshly-excised at $p = 0.05$.
[a]August (1985) ovules cultured one month.
[b]October (1985) ovules freshly-excised and fixed.
[c]September (1985) ovules cultured one month.
[d]February (1986) ovules freshly-excised and fixed.
[e]January (1986) ovules cultured one month.
[f]March (1986) ovules freshly-excised and fixed.
[g]February (1986) ovules cultured one month.

possesses validity. Ryczkowski (1985) has employed the technique to assess the respiration rates of ovules derived from higher plants, Haemanthus katherinae and Aesculus hybrida, other than pines. In addition, Dickinson (1967) employed manometry to determine whether cultured lily pollen, the male gametophyte, could respire. As seen within Figure 2, both freshly-excised and either one or six-month-cultured ovules could respire, the respiration rate of cultured ovules being lower than that of freshly-excised ovules. The respiration rate of cultured ovules also decreased with period of culture. This could be further confirmed by examining both the levels/activities of the glycolytic and TCA cycle intermediates and/or enzymes. Nevertheless, the respiration data provide support that loblolly pine ovules can be maintained in culture.

Additional support that ovules can be maintained in culture arises from the microspectrophotometric quantifications of azure-B, ninhydrin-Schiff and Feulgen stain intensities for cytological sections of both freshly-excised and cultured ovules (Table 2). Azure B was employed mainly to stain RNA. However, with azure B, it is possible to stain both RNA and DNA, through different metachromasia. Under the staining conditions used in this work, i.e., 0.25 mg ml^{-1} dye concentration, pH 4.0 for 2 hr at 50°C, RNA (where localized) stained purple whereas DNA stained blue. Flax and Himes (1952) described that with azure B, protein staining is negligible at pH 4.0 as demonstrated by the absence of all staining except that of localized polysaccharide esters following the specific removal of nucleic acids. In addition, Flax and Himes (1952), using a large variety of plant and animal tissues as test materials obtained evidences for the specificity of this differential stain. The nuclei appeared blue-green while both the cytoplasm and nucleoli appeared purple. The blue-green and purple staining were absent following digestion with both DNase and RNase.

It was difficult to differentiate nucleolar RNA from nuclear DNA. According to Menzies (1966) resolution of nucleoli less than 1 μm in diameter requires a high numerical aperture oil immersion lens, and a paraffin section not more than 2 μm. Also, the staining of nuclear DNA should be suppressed, that of nucleolar RNA preserved so that nucleoli can be clearly visualized free of interference of neighboring chromatin masses.

With regard to the use of Feulgen staining for microspectrophotometric determinations of DNA, certain preparations stained faintly but others did not. It is possible that some of the DNA was extracted by Haupt's adhesive. Greenwood and Berlyn (1968) mentioned that although Haupt's adhesive is most commonly used for plant tissue, it is unsuited in Feulgen cytochemistry. Greenwood and Berlyn also tested the effects of various fixatives and found Carnoy's fluid (used in this work) to be superior in that it facilitated vigorous staining and contributed least resistance to enzyme extraction of Feulgen-positive material in pine nuclei. As for ninhydrin staining, microspectrophotometric quantifications were performed on the nuclei since they displayed an affinity for the stain. This affinity is explained by the presence of histones, i.e., basic proteins in the nucleus.

Given the above regarding both the specificity of stains and the extractability of cellular chemicals during tissue preparation, various cells were then randomly selected and examined. Comparisons of the

staining intensities for azure B, Feulgen and ninhydrin between freshly-excised and cultured ovules did not reveal statistically significant differences between the two ovule conditions indicating that cultured ovules were not undergoing senescence. Finally, microspectrophotometric quantifications of azure B staining of ovules cultured with and without 1 μM IAA revealed a significantly elevated level of RNA when IAA was incorporated into the medium.

The last line of evidence suggesting that loblolly pine ovules can be maintained in culture involves comparing both cellular dimensions and cell numbers of freshly-excised and cultured ovules. Table 3 reveals that with the exception of one case both cell lengths and widths were either the same or increased for ovules cultured for one month provided that certain hormones were incorporated into the medium. In this connection, statistically significant differences in cell numbers between freshly-excised ovules and those maintained in culture were not evident.

Table 3. Comparisons of Cell Numbers Within Cytological Sections of Both Freshly-Excised and Cultured Ovules.

Ovule Condition	Cell Number
Cultured versus Cultured[1]	
Without hormone	25 ± 2[a]
With 1 μM kinetin	25 ± 2
With 1 μM IAA	27 ± 1
With 1 μM GA_3	26 ± 2
Freshly-excised versus Cultured	
Freshly-excised[2]	29 ± 1
Cultured with 1 μM kinetin[3]	26 ± 1
Freshly-excised[4]	24 ± 1
Cultured without 1 μM GA_3[5]	26 ± 1
Freshly excised	25 ± 2
Cultured with 1 μM kinetin	24 ± 3

[a]Cell counts are means and standard deviations where N = 3 for 9 cm^2 areas using 705X photomicrographs.
[b]August (1985) ovules cultured one month.
[c]October (1985) ovules freshly-excised and fixed.
[d]September (1985) ovules cultured one month.
[e]February (1985) ovules freshly-excised and fixed.
[f]January (1986) ovules cultured one month.

CONCLUSIONS

(1) Excised loblolly pine ovules with attached scale tissue can be maintained in culture either with or without hormones but their metabolic activity slowly declines with time; (2) The addition of certain plant hormones appears to enhance the ability of the ovule-scale tissue to be maintained in culture; (3) Comparison of macromolecular contents of fixed tissue of both freshly-excised and one-month-cultured ovules via microspectrophotometry shows that cultured ovules contain as much macromolecules (protein, RNA, DNA) as freshly-excised ovules, providing additional support for the conclusion that pine ovule-scale tissue can be maintained in culture; (4) Kinetin, IAA and GA_3 usually induce increases in cellular dimensions of cultured ovules.

SUMMARY

Inadequate regeneration of loblolly pine has been identified as a primary cause of the reduction of the timber resource in the Southeastern United States. A study of the reproductive development of pine is an important component of the understanding of the biology of this species. Here is reported whether ovules with attached scale tissue can be maintained in vitro. Viability measures were: respiration, macromolecular contents (MC) as well as cell size and number of both freshly-excised (FE) and cultured (C) ovules. Manometry was used to measure respiration and MC of stained sections were quantified by microspectrophotometry. Comparison of both FE and C ovules' respiration revealed that C ovules' O_2 consumption decreased with time. Staining sections with azure B plus microspectrophotometry revealed that 1 μM IAA additions to the culture medium resulted in higher RNA levels. One μM kinetin (K) or GA_3 enhanced cell lengths but 1 μM IAA, GA_3 or K increased cell widths vs media without hormone. Comparison of FE ovules with those C with 1 μM K usually and GA_3 always enhances widths but not lengths. Differences in cell number between ovules C with and without hormones and FE vs. C ovules and ovules cultured with either kinetin or GA_3 were not seen. Thus, ovules with attached scale tissue can be maintained in culture with or without hormone supplementation.

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SECTION III

BIODETERIORATION AND EFFECTS ON AIR QUALITY

BIODETERIORATION AND EFFECTS ON AIR QUALITY

INTRODUCTION

Biodeterioration was described by Llewellyn previously in Biodeterioration Research 2 (1989) as a "broad and multi-disciplinary topic." This section contains reports which exemplify this concept and bridge the science of biodeterioration with the human health and medical sciences.

Infestation, colonization, or contamination of our environment by microorganisms, whether occurring naturally or accidentally, can lead to unwanted changes in the objects or materials. Likewise, microbial products and other chemicals can lead to similar biodeterioration. On the other hand, the purposeful application of microorganisms/products or chemicals, while initially intended for perceived improvements to our quality of life, could effect the opposite result. The natural, purposeful, and accidental occurrences of microbial or chemical agents in our environment provide the potential for adverse exposures to humans.

Reports which emphasize certain public health aspects of the effects of biodeterioration and biodegradation are emphasized in this section. The applications of immunologic techniques in evaluating indoor air deterioration is presented. A by-product of firing bullets in indoor or outdoor firing ranges is airborne lead particles that contaminate the air. Inhalation and/or ingestion of the lead could lead to potential human health hazards for exposed individuals.

Descriptions of gram-negative bacteria (and their endotoxins) from raw cotton fibers, aqueous sludge samples, and cereal grain are presented. Likewise, fungal organisms are found in wood, sludge, and grain, and technological aspects of the definition of the microorganisms in environmental samples are discussed.

Human exposures to these organisms are likely to occur during occupational or avocational activities. Biomarkers of exposure to environmental microorganisms, e.g. specific antibodies, and biomarkers of effect, e.g. organic dust toxic syndrome (ODTS), are presented in this section, and document this concept.

A wide range of adverse health effects in humans can be attributed to respiratory exposures to microorganisms and related dusts. Acute effects, including inflammation, asthma, and ODTS, and chronic effects such as chronic bronchitis and hypersensitivity pneumonitis are described. Included in this section is the interaction of host lung defense mechanisms against infectious organisms as well. Public health concern is raised for better definition of the non-infectious biohazard exposures that exist for workers.

The reports in this timely and thought-provoking section should stimulate the biodeterioration/biodegradation research community to examine the public health implications of their research; to include in their experimental design questions concerning the potential impact on health; and to examine critically all aspects of purposeful application of biodeteriogens.

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Immunologic Aspects of the Evaluation of Health Problems Associated with Indoor Air Pollution

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INTRODUCTION

During the past few years, there has been increasing interest in and concern about indoor air quality. A myriad of problems are included in the expression "indoor air pollution" and it is beyond the scope of this report to discuss or even mention them all. The immunological questions associated with indoor air pollution are varied and complex. Rather than review the types of pollutants found in indoor air and what effects they might have on the immune system, I would like to present a brief review of how the immune system functions, how alteration in immune function can affect health, and how pollutants might alter the immune system.

The science of immunology seems to be in a constant state of flux. Many new and exciting discoveries are being reported on an almost daily basis, and it is difficult to keep up with all that is going on in the science. It is not necessary to review all of immunology, but what is appropriate and necessary is to go over the basics of an immune response, and how an immune response can contribute to disease.

Host resistance to disease is the result of two separable but closely connected systems. There are innate, non-specific mechanisms and there are acquired, specific mechanisms. The non-specific mechanisms are many and varied but include inflammatory reactions that are mediated by cells (neutrophils and monocytes) and proteins (complement and acute phase reactants). The acquired response is the immune response and it is distinguishable from an inflammatory response in that it show specificity and memory. The immune response is also mediated by cells (lymphocytes) and proteins (antibodies), but these react only with the specific agent that triggered their production.

The agents that trigger an immune or inflammatory response are generally foreign to the body, thus both the inflammatory and immune response can distingush self from non-self. In fact immunology has been called the "Science of self-nonself discrimination" (Klein, 1982). When a foreign substance is inhaled, it can stimulate an inflammatory reaction in the lung which can lead to immune response. The magnitude of the inflammatory reaction and the nature of the immune response is dependent upon both the nature of the inhaled material and the dose or quantity of materials inhaled. It is important to keep these somewhat simplistic concepts of immunology in mind when discussing the immunologic aspects of any problem.

Although the primary function of the immune response is thought to relate to protection, particularly resistance to infectious disease, it should be remembered that an immune response can also be a mechanism of disease. In simplistic terms, immunopathologic reactions occur when there is either over stimulation or suppression of an immune response. Over stimulation or hypersensitivity reactions can involve antibodies as is seen in allergies (e.g. ragweed hay fever due to IgE antibodies) and immune complex diseases (e.g. post streptococcal glomerulonephritis due to IgG antibodies), or they can involve cells as is seen in delayed hypersensitivity reactions (e.g. contact dermatitis). If hypersensitivity is contributing to a disease, then it is possible by in vitro and in vivo techniques to demonstrate the presence of antibodies or sensitized cells to the offending agent. Thus analysis of blood for the presence of immune reactants can provide evidence of exposure and possibly of the mechanism of disease when an immunopathologic disease is suspected.

DISCUSSION

From an immunologic prospective it is necessary to consider the nature of the pollutant, and the signs and symptoms reported by exposed individuals to determine what, if any, immunologic tests could aid in the evaluation of an indoor air pollution problem. For simplicity, I will assume that indoor air pollutant will most likely enter the body by inhalation. Thus the pollutant must be of respirable size (<10 μ mmd), and be immunogenic. The immunogenicity of a substance is a function of its molecular size and complexity and its foreignness. Aerosolized organic materials, such as from contaminated humidifiers, are potentially excellent antigens. Reactive low-molecular weight substances, for example formaldehyde and isothiocyanates, can act as

haptens by binding to normal proteins and triggering an immune response. The presence of inhalable, immunogenic substances in a given environment means that the potential for immune sensitization exists.

If the signs and symptoms are suggestive of an IgE mediated allergic disease (hay fever and/or asthma type symptoms), then tests for IgE antibodies to the offending agent can be performed. Skin testing using appropriate extracts is the most direct, sensitive method, but this procedure does require an experienced physician to administer the test and interpret the results. In vitro IgE antibodies can be detected using the radioallergosorbent test (RAST) (Johansson, S.G.O., 1978). In this assay, the antigen is coupled to a solid matrix and reacted with patients serum. The amount of IgE antibodies bound is then determined using a radiolabelled anti-IgE. The test is very sensitive and specific, and can be adapted for screening large populations. There are several variations on the RAST (e.g. using an enzyme tracer or different support matrixes), but the basic principal is the same. As with all immunologic assays the results need to be interpreted cautiously. With test for IgE antibodies in particular, one often lacks an appropriate positive control for many environmental pollutants. Thus, it is important to include non-specific binding (NSB) controls for each serum assayed to be certain that a reaction is positive. Persons with high IgE levels tend to show high NSB control values, and if this control was not used a false positive reaction would be observed. With immunoassays in general, and tests for IgE in particular, it is desirable to obtain serum from control populations that are matched for age, sex, smoking history, and geographical location.

If the signs and symptoms are more suggestive of another type of hypersensitivity reaction (fever, myalgia, malaise, or shortness of breath) then tests for the presence of IgG antibodies would be more appropriate. In general, skin testing has not proved useful in these situations, and one has little choice but to use in vitro assays. A number of simple and useful assays have been developed, and the choice is a function of the degree of sensitivity desired, and the nature of the antigen. The simplest assay is the double diffusion in gel, or Ouchterlony method (Ouchterlony and Nilsson, 1978). An antigen extract is placed in one well and patient serum in an opposing well, and they are allowed to diffuse toward each other. If antibodies are present a line of precipitation appears. While this method is not extremely sensitive, it is very useful as a screening procedure, and some authors have indicated that the insensitivity is an advantage in that only

sera containing significant levels of antibodies will be positive (Malmberg et al., 1985). A very similar procedure is the counter immunoelectrophoresis (CIEP) assay (Gordon, 1971). This is also a precipitation assay, but the antigen and antibody are driven to migrate toward each other by an electrical field. This greatly enhances the sensitivity relative to gel diffusion, and is very useful with dilute antigen solutions. With increased sensitivity there is increased need for appropriate controls and caution in interpreting the results. For example, in our lab we frequently screen for antibodies to fungal isolates, and find persons with antibodies to the broth in which the fungal isolate was cultured. In addition, extract of plant material, such as cotton dust, may contain substances which non-specifically precipitate serum proteins. Thus one needs to be careful to insure that the antibodies observed are real, and to relevant antigens. By including appropriate controls most of these problems can be overcome.

The most sensitive methods for detecting IgG type antibodies are the enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay. With these assays, it is usually necessary to dilute the serum samples and to determine the antibody titer. It is useful to determine a screening dilution that can clearly distingush known positive and negative samples, then if necessary titrate the positive samples. There is evidence that the antibody titers tend to be higher in symptomatic individuals with hypersensitivity pneumonitis than in asymptomatic, antibody positive persons (Marx and Gray, 1982). It should be remembered that a positive result only indicates that the individual was exposed to the antigen, and additional information is needed before one can be certain an immunopathological reaction is responsible for the disease seen.

Although cell mediated reactions have been implicated in pulmonary diseases due to inhaled substances ranging from farmer's lung disease to silicosis, the exact mechanisms involved are not well understood. For these reasons, tests of cellular immune function are not usually informative. As research progresses in this area potentially useful tests may be developed. Recent studies have shown, for example, that symptomatic farmer's lung patients have increased numbers of CD8 positive lymphocytes in bronchoalveolar lavage fluid (Reynolds, 1987). Such findings offer hope that the mechanism of disease may be better defined, and that assays can be developed that would be diagnostically useful.

The conditions discussed so far have concerned over stimulation of the immune system. At the other extreme, the lack of an immune

response can allow an infectious disease to occur to which the individual would normally be resistant. Such cases are referred to as immunodeficiency syndromes. Immunodeficiencies may be due to developmental problems (birth defects) or caused by infections as is seen in acquired immunodeficiency syndrome, (AIDS). Some drugs have immunosuppressive activities and can cause depressed immune responses; for example, cancer chemotherapeutic agents, drugs used to prevent transplantation rejection, and drugs used to treat autoimmune diseases can be immunosuppressive. Whatever the cause of the immunodeficiency, the result is lowered resistance to infections and the occurrence of opportunistic infections. Thus, is a pollutant is immunotoxic, or immunosuppressive, then the most likely evidence would be increased prevalence of infection. Several species of fungi that have been isolated from buildings with perceived indoor air pollution problems can produce mycotoxins, and some mycotoxins have been shown to have immunotoxic activity (Sorenson, 1986). However, it is unclear if the diseases reported were due to the direct toxic effects of the mycotoxins, or if the immunotoxic activity of the mycotoxins contributed to the problem. This is an area that is in need of further study. Beyond mycotoxins there are no reports of any inhaled, immunosuppressive compound causing disease.

One special case that is of concern to the immunologist is the immunocompromised individual's response to indoor air pollution. There have been approximately 100,000 cases of AIDS reported in the United States, of which, about 60,000 are still living. Patients with AIDS are increasing in numbers, are living longer, and are able to work longer. The hallmark of AIDS, and other immunocompromised persons are the opportunistic infections that usually are the ultimate cause of death. Thus when indoor air pollution problems are caused by microbial contamination of the heating, ventilation, or humidification systems, these individuals are exposed to potentially infectious agents, rather than allergenic or inflammatory agents.

One final comment about how the discipline of immunology and the problem of indoor air pollution may interact in the future. Several recent studies have demonstrated that immunochemical methods may be adapted for the detection and quantitation of airborne pollutants (Agarwal et al., 1981). This is particularly applicable to the subject of allergen contamination of air. Basically the concept is to use antibodies to known or suspected pollutants to detect the presence of those pollutants. In our laboratory, we have been using the RAST inhibition assay to detect aeroallergens (Lewis et al., 1988). Sera

from allergic individuals are used as a source of IgE antibodies to the allergen, and a competitive inhibition assay is used to determine the amount of allergen extracted from a filter of an air sampling device. By this technique, we can detect about one microgram of allergen protein on a filter. Others have used ELISA type procedures with similar results (Davies et al., 1983). These can be highly sensitive and specific assays, and it is the specificity that somewhat limits the utility of these tests. Unless one knows what the contaminant is, then it is impossible to know what antibody to use. In an initial survey of a building or worksite, these assays are of limited use. However, for continued monitoring of a site with a known problem these assays may prove very useful.

SUMMARY

From an immunologic perspective the questions associated with indoor air pollution, and in particular the contamination of indoor air by microorganisms, concerns the nature of the pollutant and the symptoms reported by exposed individuals. First, one must know if the pollutant or contaminant is immunogenic or allergenic, i.e. can the substance stimulate an immune response? Then based on the signs and symptoms of disease one can decide what type of immunopathological reaction might be involved. With this information in vitro or in vivo assays can be selected that will provide support for a particular diagnosis. No immunological test is by itself diagnostic, and the results need to be interpreted in light of the clinical and environmental findings. In the future the use of immunochemical assays may provide sensitive, specific, and quantitative measures of airborne pollutant levels. Such information will enhance our abilities to determine the source of the pollution and to evaluate control measures.

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Deterioration of Air Quality in Firing Ranges: A Review of Airborne Lead Exposures

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INTRODUCTION

Exposure to airborne lead in indoor (Fischbein et al., 1979; Novotny et al., 1987; Landrigan et al., 1975; Smith, 1976; NIOSH, 1975; Anderson et al., 1977; Olmez et al., 1984;) and outdoor firing ranges (Tripathi et al., 1989a,b) is a documented occupational health hazard. Deterioration of air quality in firing ranges poses a serious health threat to personnel who work in shooting ranges, both commercial and private. Airborne lead, dust, and fumes generated by the firing guns, cause an accumulation of lead in the blood of firearm instructors, maintenance workers, and shooters. A study conducted by the National Institute for Occupational Safety and Health (Anania and Seta, 1975) reported an average airborne lead concentration from nine indoor firing ranges of 2,700 $\mu g/m^3$, almost 54 times the current Occupational Safety and Health Administration (OSHA) standard of 50 $\mu g/m^3$ (OSHA, 1978). Another recent study conducted by Tripathi et al., (1989a) from an outdoor firing range reported an average airborne lead concentration of 129 $\mu g/m^3$.

There are approximately 16,000 to 18,000 indoor firing ranges in the United States and there are estimated to be about 1,178,000 people employed in law enforcement agencies (NIOSH, 1983). Many law enforcement agencies are demanding greater proficiency and accuracy in the use of handguns by their personnel. Besides being used by law enforcement agencies, handgun shooting is a popular recreational activity by the general public. This has led to an increase in the construction and use of indoor and outdoor firing ranges. Indoor shooting ranges offer several advantages over outdoor shooting ranges such as protection

from the weather and use of the facility around the clock. However, these ranges can and do present health hazards in the form of lead poisoning due to improper ventilation control.

During the 1960's and 1970's, the hazard of lead poisoning has been well-documented for indoor firing ranges. Several studies (NIOSH, 1975; Anania and Seta, 1975; Landrigan et al., 1975) indicated that numerous indoor ranges had lead dust and fume levels above the minimal acceptable (50 $\mu g/m^3$) levels (OSHA, 1978). The process of removal of airborne lead by engineering control (ventilation air systems) in the indoor firing ranges proved to be costly. Thus, numerous ranges were closed.

Presently, the general consensus is that outdoor firing ranges are safe from hazards of lead dust and fumes. The common opinion is that climatic conditions on the outdoor ranges dispersed the toxic lead dust and fumes. The study conducted by Tripathi et al. (1989a) provided evidence against this accepted belief. The range studied by Tripathi et al. (1989a) is shown in Figure 1. It is situated in a low-lying area which reduces the air flow across the range causing poor dispersion of the lead fumes. The range has four firing points and four firing lanes. A sand trap, to collect the spent lead, is located beneath the steel backstop, which is tilted at a 45° angle to the floor. The site is characterized as a pavilion with open sides and an "A" frame roof.

SOURCES OF CONTAMINATION

Airborne lead in indoor and outdoor firing ranges is generated by many sources during the firing of weapons. One source is the bullet primer which contains approximately 25-30 milligrams of material of which approximately 35% is lead styphnate and lead peroxide. The lead styphnate is a highly explosive compound used to initiate the combustion of gunpowder in the cartridge. Other sources of lead generation are from vaporization (due to the heat of explosion) and fragmentation of the projectile (due to cylinder and barrel misalignments and due to gaps from wear and manufacturing tolerances) as it passes through the weapon after being fired. The 2000° F temperature generated at firing causes the gases to expand creating a pressure build-up of approximately 18,000 to 20,000 psi in the cylinder that terminally blows the dust and fumes from the weapon, much of it at right angles to the direction of fire. Another source of lead contamination is from fragmentation of the bullet as it

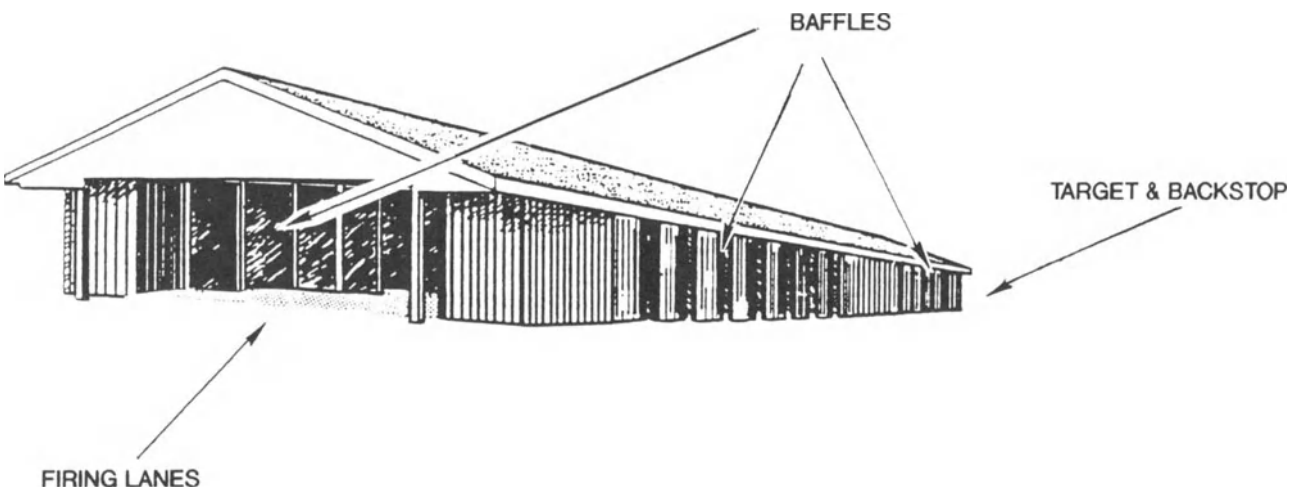

Figure 1. A Generalized Sketch of a Typical Covered, Outdoor Firing Range in Commoun Use by Law Enforcement Officers.

strikes the bullet trap. The problem of personal exposure from this source is believed to be minimal since the distance between the shooter and the bullet trap is normally 75 feet or more away.

OCCUPATIONAL HEALTH EXPOSURE CRITERIA

Present day occupational controls are directed to protection of both the worker and his family, and the environment outside of the workplace. Legislation in the United States is directed to four levels of control: air within the workplace, blood lead (PbB) levels of workers, emission of lead from the workplace, and discharge of industrial effluents. The current OSHA standard for inorganic lead in air is 50 $\mu g/m^3$ calculated as an 8-hour TWA for daily exposure (OSHA, 1978). The standard places significant emphasis on the medical surveillance of all workers exposed to levels of inorganic lead above the action level of 30 $\mu g/m^3$ TWA. The action level initiates several requirements of the standard, including periodic exposure, monitoring, medical surveillance, training, and education. The physician plays an important role in this surveillance program and in the operation of the medical removal protection program. For example, if an employer's initial determination shows that any employee may be exposed to over 30 $\mu g/m^3$, air monitoring must be performed every six months until the results show two consecutive levels of less than 30 $\mu g/m^3$ (measured at least seven days apart). The standard also dictates that workers with PbB levels greater than 50 $\mu g/dl$ must be immediately removed from further lead exposure. The affected employee must be removed from further lead exposure until the PbB concentration is at or below 40 $\mu g/dl$. With this standard, yearly average PbB levels should be 40 $\mu g/dl$ or lower. Removed workers have protection for wage, benefits, and seniority for up to 18 months until their blood lead levels decline to below 50 $\mu g/dl$ and they can return to lead exposure areas.

HEALTH HAZARDS OF FIRING RANGES

All individuals have a certain amount of lead in their bodies as a result of exposure from natural and man-made sources. Lead enters the body from inhaled air, ingested lead dust, food and beverages. For non-occupationally exposed individuals, ingestion from food and water contributes to the majority of the body burden for lead. Under normal exposure conditions, the body is able to store some lead that enters the body and excrete the balance without adverse health effects. But, when a

person is over-exposed either accidentally or occupationally to high concentrations of lead, this sensitive balance is upset and symptoms of lead toxicity may result.

The major route of absorption for inorganic lead in firing ranges is through inhalation of lead dust and fumes. A secondary route of absorption may be from ingestion of lead dust deposited on food, cigarettes, or other objects. Percutaneous absorption of inorganic lead is considered less significant. When lead is inhaled, it is estimated that the deposition rate in the lung of the human adult is between 30 to 50 percent, depending in large part on the particle size and ventilation rates (Kehoe, 1961a,b,c; Gross, 1981; Lauwyers, 1983). Large particles can lead to higher deposition rates in occupational settings, but much of this deposition occurs in the upper respiratory tract, where it is eventually moved to the gastrointestinal tract (GI) by ciliary action and swallowing. It appears that smaller particles, which become deposited in the lower regions of the respiratory tract, are completely absorbed, thereby making the exposure to lead fume a much greater exposure hazard (Rabinowitz et al., 1977).

The contribution of ingested lead to blood lead levels should not be overlooked in the occupational setting. Unless good housekeeping and personal hygiene practices are strictly enforced, ingested lead could be the major contributor to elevated blood lead levels. It is estimated that in adults, 10 to 15 percent of the lead ingested will be absorbed into the blood stream (Rabinowitz, 1974). This absorption rate can increase to as high as 45 percent under fasting conditions (Heard and Chamberlain, 1982).

The portion of the ingested lead that is not absorbed passes through the GI tract and is eliminated in the feces. Lead that enters the bloodstream, but is not retained, is excreted through the renal system and GI tract. Human metabolic balance studies have shown that short-term lead excretion in adult-human amounts to 50 to 60 percent of the absorbed fraction (Rabinowitz et al., 1976). The remaining amount moves primarily to the bone, where a portion of it will be excreted over time.

Lead will accumulate in the body with age, mainly in the bone. It is estimated that approximately 95 percent of the total body burden of lead is lodged in the bone (Barry, 1975). Bone lead is the most inert pool of lead in the body, but its accumulation in the bone can lead to elevated blood lead levels for long periods after the occupational exposure has ceased. Recent studies have shown that the decline in blood

lead levels of workers placed on medical removal is correlated with the duration of exposure (O'Flaherty et al., 1982).

The health hazards associated with exposure to lead in firing ranges can range from relatively mild, reversible effects on the various body systems to that of permanent damage, and chronic disease. Within recent years, many new methods have become available for measuring the various biochemical and physiological changes that occur as a result of a person's exposure to lead. The blood lead test is one measure of the amount of lead in the body and is the best available measure of recent lead absorption. Adults not exposed to lead at work usually have a blood lead concentration less than 30 μg/dl; the average is less than 15 μg/dl (Muhaffey et al., 1982: Annest et al., 1983).

Absorbed lead can affect the red blood cells, the kidneys, the central and peripheral nervous system, and the reproductive system. Recently, lead has been shown to be carcinogenic and mutagenic in animal studies (EPA, 1986). Some common symptoms of chronic overexposure include weakness, excessive tiredness, irritability, loss of appetite, metallic taste in the mouth, anxiety, constipation, digestive colic, nausea, pallor, insomnia, headache, muscle and joint pain, soreness, high blood pressure, fine tremors, numbness, dizziness, hyperactivity, mental deficiency or slowed reaction times.

Hematological Effects

More than 95% of the lead in blood in humans is associated with red blood cells. There are at least two compartments for lead in red blood cells, one associated with the membrane and the other with the red blood cell proteins, particularly hemoglobin. Lead inhibits the body's ability to make hemoglobin through its antagonism of iron and its interference with several enzymes in the heme pathway. Decreased production of hemoglobin leads to decrease red blood cells shown clinically as anemia which is the most serious hematological outcome of lead toxicity.

Renal Effects

Lead causes tubular dysfunction, with ultra-structural changes in the mitochondria, which results in (Fanconi's syndrome) aminoaciduria, glycosuria, and phosphaturia. It occurs with relatively short term exposure and is reversible after successful chelation therapy.

Neurological Effects

Neurological effects in adults exposed to high lead levels are more

likely to be evident in the peripheral nerves. The peripheral effects are usually reported as limited to the motor nerves producing extensor muscle weakness (foot and wrist drop); however, sensory deficits have also been observed (Brown, 1975). Considerable evidence exists that peripheral nerve dysfunction occurs in adults at PbB levels as low as 30 μg/dl (Seppalainen and Hernberg, 1980, 1982). Segmental demyelination and possibly axonal degeneration follow lead-induced Schwann cell degeneration (Lampert and Schochet, 1968). However, histological studies of nerves of humans with relatively low levels of lead exposure show no overt evidence of segmental demyelination but there may be changes in the axon membrane itself (Buchthal and Behse, 1979).

Blood lead levels above 120 μg/dl in adults have been associated with acute toxicity of the central nervous system (CNS). Manifestations include coma, convulsions, irreversible intellectual and behavioral impairment, and even death. Lead encephalopathy is the most serious effect of lead poisoning in acute lead intoxication. Subtle alterations in CNS function have been noted at lead exposures too low to produce clinical symptoms. One study found a correlation between lead uptake and physiological performance in adult workers with blood lead levels below 70 μg/dl.

Reproductive Effects

Lead is toxic to the reproductive system in both male and female workers. Severe lead toxicity has long been known to cause sterility, abortion, and neonatal mortality and morbidity. In the nineteenth and early twentieth centuries, women in lead occupations recognized lead as an abortifacient and male workers employed in lead-related industries had a high incidence of sterile marriages. Lancranjan (1975) reported that fertility of lead workers with blood lead levels above 40 μg/dl was decreased and this was correlated with an increased frequency of hypospermia (decreased number of sperm) and astherospermia (sperm with decreased motility) or teratospermia (malformed sperm). Premature fetal membrane rupture occurs in term and preterm infants in mothers whose PbB levels were 26 μg/dl (Fahim et al., 1976). Normal deliveries occurred in mothers whose blood lead levels were 14 μg/dl and below.

Chronic lead exposure is associated with infertility and fetal damage in pregnant women. The fetus has a higher risk of toxicity to lead than other people, particularly effects on the developing nervous system because it has been shown that lead crosses the placenta and the fetal blood lead approximates that of the mother at birth. Precise

levels of risk in terms of maternal or fetal PbB levels have not been determined with precision. Recently, a prospective epidemiologic study of children exposed to lead in utero and followed longitudinally through early childhood noted that lower IQ scores were correlated with higher umbilical cord blood lead, even for cord blood lead levels as low as 15-20 μg/dl (Bellinger et al., 1987). Furthermore, the uptake of lead by the fetal brain is not readily reversed by lowering blood PbB levels. There may be no threshold limit at which adverse effects could not occur in the course of development of the human fetus. One study has reported an association between risk of minor congenital malformations in humans and lead levels in blood from the umbilical cord; the increase occurs within the ranges of umbilical cord lead values found in the general U.S. population (Needlemann et al., 1983).

Lead and Hypertension

The relationship between lead and hypertension has long been suspected but has been tenuous at best, even among workmen with excessive exposure to lead (Cramer and Dahlberg, 1966). Recently, however, the potential relationship between lead and elevated blood pressure received new attention with the finding of a statistically significant relationship ($P<0.01$) between systolic and diastolic blood pressure and blood lead levels (Pirkle et al., 1985; Harlan et al., 1985) from data obtained from the Second National Health and Nutrition Examination Survey (NHANES II). Experimental support for this finding is largely from animals exposed to small increases in lead for long periods of time. The cellular basis for this effect of lead is not known, but most studies have focused on the renin-angiotensin system (Victery, 1983).

RECOMMENDATIONS FOR HAZARD CONTROL

To reduce and/or eliminate the health hazards associated with firing ranges the following control measures and work practices are recommended. These control measures can be used separately or combined to produce lower exposures.

Engineering Controls

The most important control measure is a good mechanical ventilation system. For individual ranges, the supply of tempered fresh air with some form of draftless distribution over the firing range and exhaust ventilation from the target end of the range is recommended. This is used as a method of dispersing air contaminant with air flow (Anania and Seta, 1975).

Substitution of Jacketed Bullets

Some form of jacketed ammunition such as nylon, zinc, or copper can be used (Tripathi et al., 1989b; Fischbien et al., 1980; NBS, 1977; NIOSH 1983). The cost of clad or jacketed bullets is high but in the long run may be less than that of operating a ventilation system. Zinc bullets are known to "bounce back" from the bullet trap in some ranges, causing safety hazard. Copper jacketed bullets produced the lowest concentrations of airborne lead. Therefore, they should be used as a substitute for the currently used conventional, nonjacketed bullets for reducing the lead exposures (Tripathi, 1989b; NIOSH, 1983; NIOSH, 1975).

Personal Protective Equipment

Respiratory protection is the last means of protecting employees exposed to hazardous levels of lead. Respirators must be worn through out the work shift and must be approved by NIOSH/MSHA if the ventilation is inadequate. These respirators offer the proper protection for high lead exposures.

Other Industrial Hygiene Control Measures

Good housekeeping standards, in order to prevent contamination caused by redispersal of settled dust, are recommended. A schedule for cleaning every part of the facility should be established. Vacuuming is the preferred method of cleaning up the dust, with sweeping permitted only where vacuuming is in effective. In order to prevent the lead dust from reentering the facility, emptying the vacuum into a sealed container outside the range is recommended. Surfaces of walls and floors should be cleaned by HEPA (High Efficiency Particulate Air filter) vacuuming or wet mopping. This filter is capable of trapping and retaining at least 99.97 percent of all monodispersed particles of 0.3 micrometers in diameter or larger. Any type of dry sweeping, even with dust suppression compounds is not recommended. Eating, drinking, and smoking in the range should not be allowed. This will minimize any potential ingestion of lead. All range personnel should wash their hands, arms, face, and neck after firing exercises or after any maintenance work before eating, drinking, or smoking. These activities should be restricted to a specially-designated lunch room. Showering at the end of the work day before going home is an excellent idea and should be mandatory if the range has a lead level at or above the current standard of 50 $\mu g/m^3$. Maintenance of the bullet trap can be extremely hazardous because of high lead dust concentrations. Therefore, for any cleaning, repairing, or reclaiming lead in the bullet trap, a NIOSH/MSHA approved respirator for the removal of lead dust and fumes must be worn.

SUMMARY

This review documents and describes one of the health hazards found in many indoor and outdoor firing ranges. The concern of this health hazard is the concentration of lead particles in the air of these ranges, and the effect of this exposure on user and employee health. Major sources of such exposures are conventional, non-jacketed lead bullets. Inhalation and ingestion of lead dust and fumes are the route of lead exposure in firing ranges. Adult humans absorb about 30 to 50% of the lead deposited in the respiratory tract and only 10 to 15% of the lead deposited in the gastrointestinal tract. Absorbed lead can damage the red blood cells, the kidneys, central and peripheral nervous systems, and the reproductive system. Recommendations dealing with engineering controls, substitution of jacketed bullets, personal protective equipment, and other industrial hygiene protective measures are reviewed. It is hoped that the dissemination of this and relevant information may assist in reducing or eliminating health hazards associated with the air quality of indoor and outdoor firing ranges.

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A Review on the Kinds of Potentially-Byssinogenic Gram-Negative Bacteria that Occur on Raw Cotton Fiber

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INTRODUCTION

Byssinosis is a pulmonary problem experienced by some workers in areas of textile mills where cotton, flax, or other fibers are being mechanically processed prior to spinning. It has long been associated with dust in the air of the mill. As it occurs in U.S. cotton mills, byssinosis has been attributed at least in part to a gram-negative endotoxin-bearing bacterial component of the dust dispersed from fiber into air (Jacobs and Wakelyn, 1988). Bacteria, including gram-negatives, have been shown to grow on the fiber in open bolls before harvest; such growth occurs especially during rainy weather (Simpson and Marsh, 1986; Simpson et al., 1987b). Bacterial growth on fiber is minimal in the San Joaquin Valley of California, where the weather is exceptionally dry in the period between boll opening and harvest (Simpson and Marsh, 1985). Until recently, the gram-negative bacteria on commercial cotton fiber have been only incompletely identified. We briefly review here the identification of such bacteria and discuss practical implications of these findings in relation to the byssinosis problem.

RESULTS FROM THE LITERATURE SUMMARIZED

Clark et al. (1947) reported that fiber in cotton bolls which had opened prematurely in northern Texas and in Oklahoma as a result of frost turned brownish in color and was contaminated with large numbers of bacteria identified to consist almost exclusively of Aerobacter cloacae (= Enterobacter cloacae). The fiber was thin-walled; it was regarded as inferior in usefulness for spinning and was, therefore, made into

mattresses. Workers who handled the fiber during the making of the mattresses exhibited episodes of breathing difficulty.

Rylander and Lundholm (1978), after observations on a large number of raw cotton samples, recorded in a pioneering paper that Enterobacter agglomerans (also known as Erwinia herbicola) and Pseudomonas syringae were found on almost all samples, while Agrobacterium spp. were found on about 60% of them. Klebsiella and/or Enterobacter cloacae occurred in some samples, as did also Acinetobacter calcoaceticus, Pseudomonas stutzerii, Pseudomonas maltophila, Pseudomonas fluorescens, and Flavobacterium spp. Small numbers of unidentifiable bacteria occasionally were found.

Millner et al. (1982) noted the occurrence of Enterobacter agglomerans. Klebsiella pneumoniae, Klebsiella ozaenae, and Enterobacter sakazakii on samples of commercially harvested fiber.

Millner et al. (1984) reported again on identifications of gram-negative bacteria from raw cotton. They indicated an "absence of P. syringae and the presence of a Pseudomonas-like bacterium which caused an apparent displacement of E. agglomerans from a position of dominance."

Millner et al. (1985) later published observations on bacteria from cotton dusts. They reported that "In contrast to several previously published accounts--only a few of the dusts examined in our studies had E. agglomerans as a dominant bacterium."

Fischer and Sasser (1987) indicated that they also had observed the presence of E. agglomerans and P. syringae on raw cotton fiber.

Lacey and Lacey (1987) isolated and identified bacteria from the air of cotton mills. They indicate that "The predominant types were Gram-negative yellow rods, mainly Enterobacter agglomerans ... but Enterobacter cloacae, Flavobacterium spp., Acinetobacter calcoaceticus, Alcaligenes spp., Pseudomonas spp. and cocci were also isolated."

Simpson et al. (1987a), in observations on raw cotton collected from plants near Florence, SC, and College Station, TX, recorded that they had found E. agglomerans, P. syringae, and the Pseudomonas-like Ve-2 (Flavimonas oryzihabitans) on the fiber.

Berni et al. (1988) reported that E. agglomerans was a major component of the gram-negative population detected on cotton fiber from plants during weather exposure at New Orleans, LA.

DeLucca et al. (1988) isolated bacteria from cotton fiber taken from plants grown experimentally at New Orleans, LA. They found E. agglomerans and Pseudomonas spp. to be exceptionally high in frequency of occurrence. (Refer to their Table III.)

Simpson et al. (1989) have written a paper which reports the most

extensive effort to date to identify gram-negative bacteria on commercial raw cotton. This work involved 2990 isolates of gram-negative bacteria from 702 samples of fiber from the crop of 1987 in the Texas-Oklahoma and Western cotton growing regions. The organisms most commonly encountered were E. agglomerans, E. cloacae, Pseudomonas putida, P. syringae, and members of the Pseudomonas-like Ve group. Several other gram-negatives were also found but only in low or much lower numbers. No clear qualitative differences in the make-up of the gram-negative population from one location to another could be claimed from the data. Pseudomonas aeruginosa, well known as a secondary opportunist in human infections (Bailey and Scott, 1974, p 163), was detected, but infrequently.

DISCUSSION

The literature summarized here presents a background comparison profile of the kinds of gram-negative bacteria frequently found on U.S.-produced raw cotton fiber. This profile could be used in any future episode of unexpected or unexpectedly severe acute pulmonary dysfunction in a U.S. cotton mill for comparison with the gram-negative bacteria on the byssinogenic fiber being processed.

Evidence from the literature indicates that some of the same gram-negative bacteria which tend to be dominant on commercially produced cotton fibers are also very frequently found in high numbers on leaves and seeds of other plants. The organism identified here as Enterobacter agglomerans is, in fact, known among higher-plant scientists as Erwinia herbicola and under the latter name is well known as an epiphyte of frequent occurrence on above-ground surfaces of many plants (Ewing and Fife, 1972). According to Blakeman (1982) "Erwinia herbicola, a yellow pigmented organism, has been isolated frequently from flowers and leaves of fruit trees---"; they also stated that "Strains of Pseudomonas fluorescens occur widely on leaf surfaces." Blakeman and Fokkema (1982) noted that "In addition to saprophytic bacteria, pathogenic bacteria, e.g., Pseudomonas syringae pv syringae, P. syringae pv morsprunorum, P. syringae pv glycinae, Erwinia amylovora, and E. carotovora can live in a non-pathogenic epiphytic phase on foliar surfaces." Loper and Lindow (1987) stated that "many strains of fluorescent pseudomonads are important ... as foliar epiphytes,..."; they indicated that pathovars of Pseudomonas syringae "... are bacterial epiphytes prevalent on leaf surfaces." Kremer (1987) found Erwinia herbicola (E. agglomerans) and Flavobacterium to be exceptionally frequent in occurrence on seeds of higher plants. Perombelon (1981) has noted that E. herbicola on pear and apple tree surfaces may antagonize the fire blight pathogen E. amylovora .

Holmes et al. (1987) have proposed that the names Chryseomonas luteola and Flavimonas oryzihabitans be given to the Pseudomonas-like Ve-1 and Ve-2 groups of organisms, respectively.

Baca and Moore (1987) reported that "Populations of fluorescent pseudomonads were isolated from all grass samples collected at four nursery sites in western Oregon. Mean populations averaged greater than 10^6 cfu/g of fresh tissue in both cereal ryegrass and roadside grass samples and exceeded 10^9 cfu/g in standing fields of sudangrass." Leben (1965) reviewed many papers reporting the presence of bacteria, both saprophytes and potential pathogens on plant leaves, including various species of Pseudomonas. Hirano and Upper (1983) summarized results from numerous papers reporting that bacteria with pathogenic possibilities may multiply greatly without causing disease on the surfaces of leaves. Included among such bacteria were strains of P. syringae.

The fungi that occur on field-weathered cotton fiber, like the gram-negative bacteria, comprise a population of only a few genera and species (Simpson and Marsh, 1983); with these organisms and with the gram-negative bacteria, the environmental situation is thought to exert a powerful influence limiting the kinds of organisms that can survive and grow.

SUMMARY

Many investigators have agreed in recent years that endotoxin-bearing gram-negative bacteria that are dislodged from raw cotton fiber into mill air during mechanical processing are probably important causative factors in byssinosis among millworkers. Until recently, however, the gram-negative bacteria that occur on U.S.-produced raw cotton fiber have been incompletely identified. We review here the results of published identifications of such bacteria. Most frequently encountered among samples examined at USDA-Beltsville have been E. agglomerans, E. cloacae, P. putida, P. syringae, and members of the Pseudomonas-like Ve group. Somewhat less frequently seen were P. aeruginosa, P. fluorescens, P. paucimobililis, P. pickettii, P. stutzeri, P. alcaligenes, P. pseudoalcaligenes, and P. vesicularis. Occasionally or infrequently encountered were species of Acinetobacter, Flavobacterium, and Klebsiella. The gram-negative bacterial population on the fiber thus has appeared to consist mainly of a very limited number of species. Practical implications are discussed and comparisons are made with the kinds of bacteria detected by others on senescent leaves and on seeds of various plants in above-ground field exposures.

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Microbiological and Serological Studies of an Outbreak of "Humidifier Fever" in a Print Shop

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INTRODUCTION

The aerosolization of microorganisms can occur in a variety of environments, and in certain situations can induce pulmonary reactions in exposed persons. One example of this situation occurs in the operation of humidification systems. The pulmonary reaction is thought to result from the microbial contamination of the water used in the humidification system coupled with the dispersion of the contaminants by the air system being humdified. This aerosolization of microbes and their metabolites can cause disease by infection as is seen in "Legionnaire's Disease" (Basich, et al 1980), by inducing an allergic and/or asthmatic reaction (Solomon, 1974), or by inducing an acute, febrile pulmonary reaction often referred to as "Humidifier Fever" (Parks, 1982).

Humidifier fever is characterized by onset of symptoms (cough, fever, shortness of breath) 4 to 8 hours after exposure, but is not well defined with respect to the mechanism of disease or the etiological agents involved. In some of the reports in the literature there is good evidence that the clinical findings are consistent with a diagnosis of hypersensitivity pneumonitis (HP) (or extrinsic allergic alveolitis) (Banaszak, 1970). This is an immunologically mediated inflammatory reaction, and precipitating antibodies to the microbial contaminants are often found in sera of the symptomatic individuals. Such antibodies are also found in the sera of asymptomatic, exposed individuals. Thus the

presence of antibodies is not diagnostic, but rather indicates previous exposure. In addition, a very similar cascade of symptoms has been reported in which there wereno precipitating antibodies. This illness may represent a non-allergic, systemic reaction to microbial toxins contained in the aerosol and has been given the name "Organic Dust Toxic Syndrome" (ODTS) (doPico, 1986). In both HP and ODTS, numerous microorganisms have been implicated as causative agents including fungi, amoebae, and gram-negative bacteria. It is important to distinguish between HP and ODTS because both the short-term therapy and the long-term sequelae are different.

During the summer of 1988 we were asked to assist in the investigation of an outbreak of humidifier fever that occurred in a print shop in Vermont. The results of that investigation are the subject of this report.

MATERIALS AND METHODS

Description of the Outbreak: In early June of 1988 the Vermont Occupational Safety and Health Administration (OSHA) officials were notified by the owner of a small print shop that he and a number of his employees had become ill with an acute febrile illness. He reported that the ventilation system, designed to maintain negative pressure in the press room, was not functioning, and that because of the unusually dry weather that the press room humidifier had automatically come on. The atomizer-type humidifier had not been used or cleaned for approximately three months. Two days after the outbreak the Vermont OSHA officials conducted an environmental inspection of the facility looking for a source of toxic fumes, and obtained samples of the water and sludge from the humidifier. The samples from the humidifier were cultured for bacteria, thermophilic actinomycetes, and fungi on appropriate media at 25, 35, and 55 C, and the isolated organisms identified.

A case was defined as an exposed individual with two or more of the following symptoms: myalgia, fever or chills, cough, and chest tightness or shortness of breath. Of 28 workers in the print shop 16 (57%) met the case definition. Fever and myalgia were the most common symptoms being reported by 94% of the cases. The onset of symptoms ranged from 5 to 13 hours and lasted from 2 to 24 hours. Serum samples were collected from 29 of the exposed workers 5 days after the outbreak and again 28 days later. Sera was also collected from 7 non-exposed laboratory workers for control purposes. NIOSH was asked to assist in

this investigation, and we received samples of the sludge, the isolated microorganisms, and the serum samples.

A total of 9 species of bacteria were isolated from the water and sludge samples. All of the bacterial isolates were gram-negative organisms and were identified with the API^{R} System 20 E (for enterobacteria) and NFT (for non-fermenting bacteria) (API Analytab Products, Plainview, NY). One isolate failed to grow on subculture and was not fully identified. The bacterial isolates were maintained on tryptic soy agar (TSA) slants. Bacterial antigens for serologic studies were prepared by culturing the isolates for 24 to 48 hours in TSA broth, transferring 1.0 ml of the suspension to TSA plates and 24 or 48 hours later washing the bacteria from the plate with carbonate buffer (pH 9.5). The bacterial suspensions were diluted in the same buffer to a turbidity of 0.5 at 450 nm and used for serologic analyses (see below).

Three fungal isolates were obtained and were subcultured on malt agar. The isolates were identified as Fusarium species by morphological criteria, but were not further identified. The fungal isolates were transferred to malt broth and cultured at 37 C for four to six weeks until confluent, mature cultures were obtained. The mycelial mats and spent media were homogenized and antigen extracts prepared as previously described (Olenchock, et al, 1989b).

From the sludge sample one amoeba isolate was obtained, and successfully cultured in cell-free media (yeast extract-glucose broth containing 10% fetal bovine serum, 45 ng/ml L-methionine, 100 units/ml penicillin and 100 µg/ml streptomycin). The isolate was identified as an Acanthamoeba but not speciated. The isolate was cultured for 8 weeks at 30 C in the cell-free medium. The amoeba culture was disrupted by sonication, clarified by centrifugation, and used as an antigen extract in the serologic assays described below.

The serum samples were assayed for antibodies to the bacterial isolates by an enzyme-linked immunoassay (ELISA) (Voller and Bidwell, 1986) modified for whole bacteria. Briefly, the bacterial suspensions in carbonate buffer were placed on the wells of Immunolon I ELISA plates and allowed to adhere for one hour at room temperature. The plates were washed six times with buffer (0.1 M phosphate buffered saline containing 0.5% Tween-20), sera diluted 1:100 was added, and the plates incubated for one hour as before. Polyvalent antisera to human immunoglobulins conjugated to horseradish peroxidase (Sigma, St. Louis, MO) was added to the wells, the plates incubated as before, a solution of H_2O_2 and substrate (ABTS, Sigma) added to the wells, and the absorbance at 410 nm

was determined. Known positive and negative control sera were included in each assay. The mean absorbance of the seven serum samples from non-exposed controls was determined, and a sample was considered positive if the absorbance value was at least twice the mean of the non-exposed controls.

The sera were tested for precipitating antibodies to the fungal and amoeba isolates by counterimmunoelectrophoresis (CIEP) (Gordon, et al., 1971). One or more line of precipitation was considered positive. The sera were also evaluated for antibodies to the fungal and amoebic isolates by the ELISA as described above except that a 1:50 dilution of the extracts was used to coat the ELISA plates.

RESULTS

The environmental investigation of the print shop revealed that the water reservoir and baffles of the humidifier were covered with a brownish-gray sludge. No source of metal or polymer fumes that could have caused the reported symptoms was found. Thus the humidifier appeared to be the most likely source for the offending agent(s), and samples of the sludge were taken for laboratory analysis. Of note, Legionella pneumophila was not found in the sludge, and serologic analysis of the sera from the workers revealed no antibodies to this organism in the acute or convalescent serum samples.

Table 1. Microbiological Analysis of Humdifier Sludge.

Fungi - three Fusarium strains
Amoebae - Acanthamoeba spp.
Gram Negative Bacteria -
Enterobacter cloacae
Pseudomonas luteola
Pseudomonas acidovorans (2 strains)
Flavobacterium spp.
Alcaligenes spp.
Acinetobacter calcoaceticus
CDC group IV C-2

The results of the microbiological analysis of the sludge sample are shown in Table 1. The fungal isolates were all identified as Fusarium species by morphological criteria, but the pigmentation of the colonies was such that they appeared to be three distinct species or strains. The bacterial isolates were all gram-negative bacteria, but only one of the 8 isolates examined was an enterobacterial species (E. cloacae). The other seven were non-fermenting, mostly oxidative bacteria which showed weak activity in conventional biochemical assays, and the results should be considered as tentative.

The prevalence of antibodies to each of the microorganisms isolated was determined by the ELISA assay, and the results are shown in Table 2. As can be seen the most prevalent antibodies were to Alcaligenes and Acinetobacter, and to the fungal isolates. The prevelance of antibodies was not increased between the acute and convalescent sera indicating that infection or sensitization did not occur as a result of the

Table 2. Prevalence of Antibodies in the Sera of Workers Exposed to Humidifier Sludge Aerosol As Determined by ELISA.

Organism	Sera from Exposed Workers		
	Acute N=29	Convalescent N=28	Control N=7
Enterobacter cloacae	0[a]	0	0
Pseudomonas luteola	7 (24)	3 (11)	0
Pseudomonas acidovorans 1	1 (3)	3 (11)	1 (14)
Pseudomonas acidovorans 2	0	2 (7)	0
Flavobacterium spp.	0	0	0
Alcaligenes spp.	18 (62)	15 (54)	1 (14)
Acinetobacter calcoaceticus	18 (62)	15 (54)	0
CDC group IV C-2	4 (14)	5 (18)	0
Fusarium spp. 1	14 (48)	3 (11)	0
Fusarium spp. 2	13 (45)	18 (64)	0
Fusarium spp. 3	15 (52)	2 (7)	0
Acanthamoeba spp.	7 (14)	3 (11)	0

[a]Number of positive samples, percent positive in parenthesis.

exposure. Two of the control sera were found to have antibodies to one organism each, and for these organisms positive was defined as twice the mean of six controls rather than the seven as was used for the other isolates.

The data was further analyzed with respect to cases versus non-cases and in terms of prevalence of antibody and the relative quantity of antibody found. Because the presence of antibodies at the time of the outbreak would indicate pre-existing sensitization, only the acute serum samples were analyzed in this manner. As shown in Table 3 the overall prevalence of antibodies was not correlated with the health status of the worker. The absorbance reading from the ELISA can be taken as a measure of the relative concentration of the level of antibody. We found no difference in the mean absorbance values between cases and non-cases, nor between cases and controls for any of the organisms isolated (data not shown). In addition, we compared the mean absorbance values for each organism and for each subject category, and found no difference between the acute and convalescent serum samples.

Table 3. Prevalence of Antibodies in the Sera of Cases and Non-Cases of Humidifier Fever As Determined by ELISA.

Organism	Cases N=15	Non-Cases N=14
Enterobacter cloacae	0[a]	0
Pseudomonas luteola	3 (20)	4 (29)
Pseudomonas acidovorans 1	1 (7)	0
Pseudomonas acidovorans 2	0	0
Flavobacterium spp.	0	0
Alcaligenes spp.	9 (60)	9 (64)
Acinetobacter calcoaceticus	10 (67)	8 (57)
CDC group IV C-2	1 (7)	3 (21)
Fusarium spp. 1	8 (53)	5 (36)
Fusarium spp. 2	7 (47)	6 (42)
Fusarium spp. 3	8 (53)	6 (42)
Acanthamoeba	4 (27)	3 (21)

[a]Number of positive samples, percent positive in parenthesis.

These results indicate that for the organisms tested there was no difference in the pattern of reactivity between the worker who became ill and those who did not.

The fungal and amoebic extracts were also analyzed by CIEP for the presence of precipitating antibodies. Bacterial antigens of gram-negative bacteria do not yield reliable results is this assay and they were not tested. As shown in Table 4 the prevalence of antibodies was much lower in this assay than was seen with the ELISA. This is most probably a reflection of the greater sensitivity of the ELISA. The sera that were positive in the CIEP assay were more likely to be sera from cases than non-cases, but by Chi-Square analysis of the data reactivity in the CIEP could not be correlated with the health status of the workers.

Table 4. CEIP Results on the Prevalence of Antibodies to Fungal and Amoeba Extracts in the Sera of Exposed Workers.

Extract	Cases (N=15) CIEP	Non-Cases (N=14) CIEP
Fusarium spp. 1	5 (33)[a]	2 (14)
Fusarium spp. 2	3 (20)	0
Fusarium spp. 3	6 (40)	1 (7)
Acanthamoeba	3 (20)	0

[a]Number of positive samples, percent positive in parenthesis.

Finally we compared the results obtained with CIEP with those obtained by the ELISA method to determine how concordant the two assays were. If the difference between the assays is a matter of sensitivity, then one would predict that the sera positive in the CIEP would also be positive in the ELISA test. As shown in Table 5, this is not necessarily the result suggesting that the assays may not be comparable; however, statistical analysis for concordance (Kappa statistic) showed that there was significant agreement ($p<0.001$) between the assays.

Table 5. Comparison of the Results Obtained by ELISA and CIEP Assays for Antibodies to Fungal and Amoeba Antigens.

Assay	ELISA		
CIEP	Positive	Negative	Totals
Positive	20	13	33
Negative	52	143	195
Totals	72	156	228

DISCUSSION

The results of this investigation indicate that the outbreak of humidifier fever in this print shop was probably due to ODTS rather than HP. The conclusion is based on the negative serologic finding reported here, as well as the high attack rate, and the symptoms reported. On the recommendation of the Vermont State Health Department, the print shop has begun a maintenance program that includes regular cleaning and disinfection of the humidifier. Since this action was taken no complaint of symptoms have been reported by the print shop workers.

The term ODTS is a relatively new name for an acute febrile illness associated with exposure to organic dusts (doPico, 1986). Previous terminology has included atypical farmer's lung (Jones, 1982), precipitin negative farmer's lung (Edwards, et al, 1974), pulmonary mycotoxicosis (Emanuel et al., 1975), silo unloader syndrome (Pratt and May, 1984), and grain fever (doPico, et al, 1982). Although a toxic reaction has not been established as the mechanism for this syndrome, the syndrome is most frequently reported after exposures to microbial aerosols which could contain a variety of toxic and proinflammatory substances. It is important to distinguish ODTS from other pulmonary diseases because there appears to be no long-term pulmonary impairment, and subjects will rapidly recover after being removed from the environment, and usually do not require therapy. Although most of the

reported outbreaks of ODTS have been associated with the farm environment or agricultural processes, it is reasonable to think that this illness could occur in an situation in which individuals are exposed to microbial aerosols. The activation of a humdifier system that was highly contaminated with fungi, amoebae, and bacteria could create such a situation.

Although many of the earlier reported outbreaks of humidifier fever have been classified as HP or allergic alveolitis, it has been recognized that often the outbreaks of humidifier fever had features distinct from classical HP, such as lack of radiological changes and lack of persistent decreases in lung function (Nordman, 1984). These differences led Nordman to propose that this condition be termed "humidifier syndrome" (Nordman, 1984). This terminology emphasizes the source of the offending agents, but does not describe the mechanism of disease. Humidifier syndrome has many of the same features as ODTS although some of the characteristics, particularly the low concentration of antigen necessary to trigger a reaction, are not usually seen in ODTS. Some of the reported outbreaks of humidifier fever have been clearly documented to be due to HP (Flaherty, et al, 1984). Thus it appears that humdifier aerosols can induce pulmonary reaction by more than one mechanism. All of the bacteria isolated from the humidifier sludge were gram-negative organisms. It is interesting to note that gram-negative bacteria have been implicated in outbreaks of HP, ODTS, and humidifier syndrome (Flaherty, et al, 1984; Olenchock, et al, 1989a, Rylander, et al, 1978).

The serologic data accumulated during this investigation indicated that antibodies to the isolated microorganisms were not associated with the development of symptoms in the exposed workers. This is consistent with previous reported outbreaks of ODTS (doPico, 1986), and indicates that the response was not antibody mediated. The comparison of the results obtained by ELISA and CIEP are of interest in that neither assay was predictive of the health status of the individual. By statistical analysis the two assays were found to be concordant, i.e. the number of samples positive in both assays or negative in both indicated that there was good agreement between the assays. The ELISA is a much more sensitive method for detecting antibodies, but that increased sensitivity means that one needs to be more cautious in interpreting the results. Although the results were essentially negative in this study, they are important in the sense that they help establish ODTS as the likely cause for this outbreak.

SUMMARY

An outbreak of humidifier fever in a print shop was investigated to determine the possible role of microorganisms in this illness. Eight species of gram-negative bacteria, three fungal species and one amoeba were isolated from the sludge of the contaminated humdifiers. ELISA and CIEP analysis of serum sample from exposed workers classified as either cases or non-cases of humidifier fever were performed. The prevalence of antibody to the microorganisms as determined by either assay was not correlated with symptomology. The results of the laboratory studies coupled with the high prevalence of disease in the exposed workers, and the symptoms reported indicate that this outbreak of humidifier fever was most likely an example of ODTS induce by aerosolized microorganisms.

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Microbial Contamination and Immunologic Reactivity of Stored Oats

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INTRODUCTION

Workers in various agricultural environments, from farms to port grain terminals, are exposed to a myriad of respiratory insults during the planting, harvesting, transport, and processing of grains (Donham, 1986). Combined exposures to airborne allergens, toxins, bacteria, fungi, their metabolites and toxins, gases, vapors, and farm chemicals compound the health problems of farm operators and farm workers, many of whom are children and young adults. These types of exposures are not unique to agricultural workers in the United States, but rather, they are global in nature, both in developed and in developing countries.

Movement of stored grains has been associated with potential respiratory hazards because of the airborne dusts that are generated during the activity (doPico et al., 1980). The wide range of signs and symptoms are both acute and chronic in nature. A recently recognized acute reaction to respiratory exposures to high concentrations of organic dusts was described as "organic dust toxic syndrome" by doPico (1986). This self-limited, flu-like illness can be precipitated in various environments where agricultural dusts are generated, and the etiology remains undefined.

We recently participated in an immunologic, microbiologic, and medical evaluation of a cluster of cases of organic dust toxic syndrome in farm workers who shoveled approximately 800 bushels of oats from a poorly ventilated storage bin (Parker et al., 1988). This report

describes the exposures to bacteria, fungi, and endotoxins as well as *in vitro* and *in vivo* immunologic studies related to the presence of specific antibodies and serum complement activation.

MATERIALS AND METHODS

Bulk oats were collected from a storage bin that was associated with a cluster of cases of organic dust toxic syndrome (Parker et al., 1988). A portion (10.0 gm) of the oats was subjected to acoustical vibration in a laboratory dust generator (Frazer et al., 1987), and respirable dust was collected either on filter cassette for endotoxin analyses or by sterile saline-filled liquid impinger for microbiological analyses.

Airborne dust samples that were collected by liquid impinger were subjected to serial 10-fold dilution in sterile saline, and 0.1 ml portions were plated onto appropriate agar medium: Tryptic Soy Agar (DIFCO Laboratories, Detroit, MI) for total aerobic bacteria; half strength Tryptic Soy Agar for thermophilic actinomycetes; MacConkey Agar for enumeration of gram-negative bacteria; and Rose Bengal Streptomycin Agar for fungi (Morring et al., 1983). The plates were incubated for the respective microorganisms as described previously (Dutkiewicz et al., 1989), and the colony forming units (CFU) were counted and reported per cubic meter (m^3) of air.

Ten predominating fungi were isolated in pure culture, and grown in a shaking culture in malt extract broth (DIFCO) at 28^{o}C for 5 days. The cultures were then subjected to shearing (Polytron; Brinkman Instruments, Westbury, NY) and sonication (Sonifier Cell Disrupter, Model 350; Branson Sonic Power Co., Danbury, CT). After the sonicates were centrifuged at approximately 1200 x g for 30 min, the supernatant fluids were filtered through a 0.45 µm pore size filter and frozen at -70^{o}C. The frozen filtrates were freeze-dried (Edwards model 12K Supermodulyo Freeze Dryer; Edwards High Vacuum, Crawley, England) and reconstituted at 25% initial volume with water for use in antibody assays.

Both airborne dust and bulk samples were analyzed for gram-negative bacterial endotoxin content by a chromogenic modification of the *Limulus* amebocyte lysate test (QCL-1000; Whittaker Bioproducts, Walkersville, MD). Sterile, non-pyrogenic plastic ware was used throughout these assays. Airborne dust collected onto a filter was extracted with 10 ml sterile, non-pyrogenic water (Travenol Laboratories, Inc., Deerfield, IL) by rocking for 60 min at room temperature. The solutions were

centrifuged for 10 min at 1000 x g, and the supernatant fluids were analyzed in duplicate for endotoxin contamination. Bulk oats (1.0 gm) were extracted in 25.0 ml of water and treated similarly.

Serum samples were collected from 10 workers. Two workers were not exposed to the high concentrations of airborne dust and were not ill, while eight workers did the shoveling and were ill. Each serum was tested for specific precipitating antibody to the bulk oat extract and to the 10 predominating fungi by counterimmunoelectrophoresis (Gordon et al., 1971). Positive controls were used for each test serum. Each serum was tested further by enzyme-linked immunosorbent assay (ELISA; Marx and Gray, 1982) for specific antibody (IgG) against 9 agents in a hypersensitivity pneumonitis panel. A titer of 1:80 or greater was considered as positive.

The extract of bulk oats was studied further by assaying the interaction of the extract with the functional human complement system in vitro. Dose-dependent consumption of complement was measured by the hemolytic tube technique in terms of CH_{50} U/ml and by the diffusion and lysis in gel technique in terms of CH_{100} U/ml (Quantiplate; Kallestad, Austin, TX). The oat extract (0.0 µl to 600 µl) was added to 1.0 ml of pooled normal human serum, and the complement consumption was measured as described previously (Olenchock et al., 1980).

RESULTS

Bulk oats were obtained from the facility where a cluster of cases of organic dust toxic syndrome was described. Because the cases occurred after the oats in the bin were shoveled, no real-time dust measurements could be taken. We therefore generated airborne dust from the bulk oats by means of acoustical vibration in a laboratory dust generator. The mass median diameter of the oat dust particles was approximately 5 µm, and the diameter at peak number concentration was approximately 1 µm. Calculation of the airborne concentration of the dust that was generated from the bulk oats resulted in a value of 39.5 mg/m^3.

Both the bulk sample and the airborne dust contained gram-negative bacterial endotoxins. The contamination of the bulk dust was 122.66 Endotoxin Units (EU) per milligram. The collected dust contained 325.71 EU/mg. Calculation of the airborne concentration of endotoxins that was generated in the laboratory resulted in 12865.5 EU/m^3.

Microbial contamination of the airborne dust is shown in Table 1. Mesophilic bacteria and thermophilic actinomycetes were counted but not identified. The ten most common fungi were isolated and identified: Alternaria sp., Aspergillus flavus, A. fumigatus, A. niger, A. terreus, A. glaucus group, Cladosporium sp., Fusarium sp., Penicillium sp., and Scopulariopsis sp.

Counterimmunoelectrophoresis of sera from both ill and not-ill workers against antigens made from the predominant fungi found only one worker who had precipitating antibodies to any of the fungal antigens. This ill worker's serum reacted positively with antigens from Aspergillus niger and A. glaucus group. This same worker and one other ill worker had detectable antibodies against the crude extract of the bulk oats. Both workers who were not ill reacted positively to the bulk oat extract also.

The results of the ELISA testing for specific IgG antibodies against antigens in a typical hypersensitivity pneumonitis panel are shown in Table 2 for ill, not-ill, and all workers. While most workers reactived positively with Micropolyspora faeni antigen, it was not possible with either ELISA or counterimmunoelectrophoresis to separate the ill from the workers who were not ill.

Both assays for hemolytic complement activation demonstrated that the extract of bulk oats contained a substance(s) that consumed functional human complement in a dose-dependent fashion (Figure 1). Relatively similar curves were generated for both the CH_{50} and CH_{100} studies.

DISCUSSION

Exposure to the myriad of dusts that are generated during the day-to-day operations of farming can result in a variety of adverse effects on the respiratory system of the exposed individuals. Acute effects including inflammation, occupational asthma, and toxin fever, and chronic effects such as chronic bronchitis and hypersensitivity pneumonitis have been reported (Rylander, 1986). Toxin fever and other acute febrile reactions to high concentrations of organic/agricultural dusts are now considered related to organic dust toxic syndrome (ODTS; doPico, 1986). ODTS occurs in a variety of different environments such as those where a farm worker or farm operator is exposed to dusts from silage, grains, and wood chips (doPico, 1986). The syndrome has been reported in a non-farm situation as well; however, the inciting material in that case was moldy straw (Brinton et al., 1987).

Table 1. Colony Forming Units (CFU) per Cubic Meter of Air From Laboratory-Generated Oat Dust.

Agent	Colony Forming Units (CFU/m^3)
Mesophilic Bacteria	
Total Bacteria	1.4×10^5
Gram-Negative Bacteria	1.5×10^3
Thermophilic Actinomycetes	1.8×10^5
Fungi	8.3×10^4

Although this illness is now recognized, the causative agent(s) has yet to be defined. It is to that end that we undertook this current investigation. The farming practice of unloading grain storage bins is a common one, and the exposure to high concentrations of organic/agricultural dusts occurred. Our efforts were directed, then, to the definition of the agents to which the farm workers were exposed.

Because of the nature of our participation in this investigation, we were not able to obtain actual airborne dusts while the workers were moving the oats. However, we did generate airborne dust from the bulk oat sample in the laboratory. The dust was predominantly in the respirable range, and the generation of 39.5 mg/m^3 was similar in magnitude to the respirable dust level found within a dusty silo (Olenchock et al., 1987). Likewise, the endotoxin concentration of 12865.5 EU/m^3 (10 EU = 1 ng in our test) was similar to the high level found in respirable dust within the silo (Olenchock et al., 1987). Toxin fever is related to exposure to airborne endotoxins (Rylander, 1986), and our study demonstrated the potential for the generation of high endotoxin levels in the air of the oat bin. Further, because the oats were agitated previously during the unloading process, the levels

Table 2. Distribution of IgG Antibodies in Workers to Antigens in a Hypersensitivity Pneumonitis Panel as Detected by ELISA

Antigen	Ill[a] (N=8)	Not Ill (N=2)	Total (N=10)
Micropolyspora faeni	5	1	6
Thermoactinomyces vulgaris-MFLD[b]	2	1	3
-H/S	0	0	0
T. sacchari	0	0	0
T. candidus-GR[b]	0	1	1
-LI	0	0	0
-KO	0	0	0
Saccharomonospora viridis	1	0	1
Aspergillus fumigatus -1	0	0	0
-6	0	0	0
-1022	0	0	0
A. niger	2	0	2
Penicillium roqueforte	0	0	0
Pigeon Serum	0	0	0

[a]Those workers reported as ill.

[b]Isolate designations.

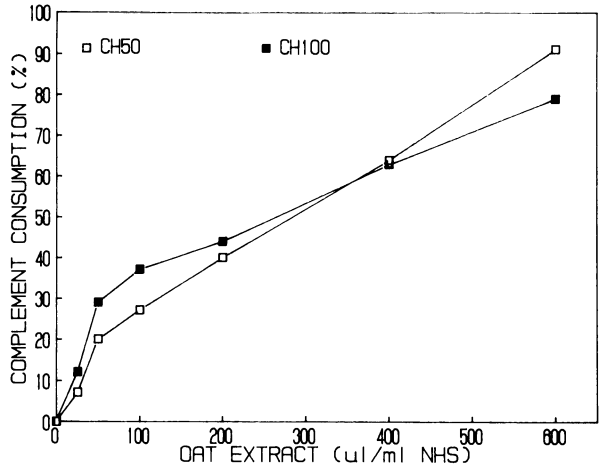

Figure 1. Dose-response Curves for Percent Complement Consumption When Oat Extract Was Added to Pooled Normal Human Serum and Tested by CH_{50} and CH_{100} Techniques.

of dust and endotoxins that were generated in the laboratory probably represent an underestimate of the actual exposure potential.

Viable gram-negative bacteria levels were likewise high (1.5×10^3 CFU/m^3), a level that exceeded a suggested hazardous level of $10^3/m^3$ (Clark, 1986). The complete role of total bacteria, viable and non-viable, in eliciting adverse respiratory responses is undefined at present.

We detected 11.8×10^5 CFU/m^3 thermophilic actinomycetes, agents associated with farmer's lung disease (Chmelik et al., 1974). This figure is approximately 2 logs lower than the number of thermophilic actinomycetes that were generated in the laboratory from surface silage from a tower silo (Dutkiewicz et al., 1989). However, it must be emphasized that the critical number of these organisms that is required to elicit an adverse biological response is unknown.

The presence of 8.3×10^4 CFU/m^3 for viable fungi is far below a suggested hazardous level of 10^7 CFU/m^3 (Clark, 1986). As is the case with levels of the other microorganisms, it must be recognized that these data just begin to examine the levels of viable components in airborne dusts in the farm environment. A systematic approach to defining the biohazards is needed.

The predominant fungi were typical of those that are related to grains and grain dusts (Lacy, 1980; Dutkiewicz et al., 1988). The absence of precipitating antibodies to the fungal antigens in all but one worker is not surprising in light of the typically negative serology that is a feature found with cases of ODTS (Brinton et al., 1987). Neither the studies with an extract of the bulk oat sample nor the ELISA studies with the hypersensitivity pneumonitis panel of antigens were able to distinguish ill workers from those who were not ill. Although our study population was admittedly small, the typically negative serology feature of ODTS is consistent with our findings. The singular exception would be our finding that 6 of 10 workers demonstrated specific IgG against Micropolyspora faeni, a common contaminant of agricultural materials (Dutkiewicz et al., 1988), thus implying that our study population was a highly exposed group.

Bulk oats that were obtained from the area of the cluster of ODTS cases contained a water soluble agent(s) capable of activating human complement in a dose-dependent fashion. This observation is consistent with our previous results of airborne oat dust that demonstrated the complement activating potential of oat dust. The efficacy of activation of human complement by oats and other grains, as well as the multi-faceted inflammatory potential of their airborne dusts (Ye et al., 1988), illustrate possible mechanisms of inflammation in the absence of specific antibody that could occur in the lung after inhalation of dusts from cereal grains.

In conclusion, our data demonstrate relatively high levels of endotoxins and microorganisms in dusts generated from bulk oats that were associated with a cluster of cases of organic dust toxic syndrome in farm workers. Antibody studies of the workers failed to differentiate the ill from not-ill individuals, but complement studies demonstrated the potentially inflammatory nature of the oats.

SUMMARY

Bulk samples of oats were obtained from Alabama where a cluster of cases of organic dust toxic syndrome occurred in workers who shoveled 800 bushels of oats from a poorly ventilated storage bin. Airborne dusts were obtained from the samples by acoustical vibration in a laboratory dust generator and analyzed for microbial contamination by standard dilution plating for total bacteria (1.4×10^5 CFU/m^3), gram-negative bacteria (1.5×10^3), thermophilic actinomycetes

($1.8x10^5$), and fungi ($8.3x10^4$). Analysis of the dust for gram-negtive bacterial endotoxins resulted in the detection of 325.71 Endotoxin Units per mg. An extract of the bulk sample consumed human complement in vitro in a dose-dependent fashion. The sera from the exposed workers were examined for antibodies against an extract of the bulk oats, antigens from predominant fungi, and standard hypersensitivity pneumonitis antigens. Evidence of exposure could be determined, although ill and asymptomatic workers could not be differentiated. The stored oats provided a source of exposure to microbial antigens and immuno-reactive materials.

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Pulmonary Inflammatory and Immunological Responses to Airborne Pathogens: A Review

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INTRODUCTION

Influenza and the associated pneumonia is the sixth leading cause of death in the United States. The economic cost in terms of medical dollars and work loss is about $5 billion a year. Deterioration of air quality in the workplace may be caused by microbiological or particulate pollution and such pollution may impair pulmonary defenses sufficiently to enhance the susceptibility to airborne infections such as influenza. Tobacco smoke has been implicated as a disease causing agent even in individuals exposed to second-hand smoke. Ventilation systems in buildings have been implicated in transmission of infectious disease including tuberculosis, measles, smallpox and staphylococcus infections. Carpeting, humidifiers, and flush toilets can harbor bacteria, pollen, fungi, and other allergens. A number of viruses with varying degrees of epidemiologic and pathologic significance may be aerosolized, contribute to air quality deterioration, and cause respiratory disease (Table 1). Influenza viruses are prone to antigenic drift and consequently humoral immunity from previous infections is often only partially effective at preventing disease. The rationale behind vaccinating with a killed or modified live virus vaccine (or prior clinical or subclinical disease) is to stimulate the production of antibodies to certain epitopes on the viral surface. These epitopes are in constant contact with the host blood antibodies and hence are subject to a great deal of selective pressure. This selective pressure tends to favor the emergence of new viral epitopes and hence loss of disease resistance. Therefore, areas with a great deal of endemic disease are conducive to emergence of new viral strains.

Table 1. Respiratory Disease Associated Viruses.

UPPER RESPIRATORY TRACT[a]	
Rhinovirus	Epstein-Barr
Parainfluenza 1-4	Respiratory Syncytial
Influenza A, B, C	Corona Virus
Herpes Simplex	
Adenovirus	
Echoviruses	
Coxsackieviruses	

BRONCHOLITIS[b]	
Respiratory Syncytial	Influenza A
Parainfluenza 1-3	

LOWER RESPIRATORY TRACT[c]	
Influenza A	Parainfluenza 1-3
Adenovirus	Measles
Herpes Simplex	
Varicella- Zoster	
Cytomegalovirus	
Respiratory Syncytial	

[a] Upper Respiratory Tract to include the pharynx

[b] Several serotypes

[c] Lower Respiratory Tract and a causative agent in interstitial pneumonia

The development of clinical disease varies with the nature of the pathogen, e.g., degree of antigenic shift, prevalence, transmissibility, infectivity, pathogenicity, and virulence. The major reason for vaccinatinating is not so much to prevent an individual from developing disease as it is to break the cycle of transmission.

It is the purpose of this review to relate, in a very general sense, deterioration of air quality by biological or physical agents to the pathogenesis of disease. To illustrate the point, bovine pneumonic pasteurellosis (a viral-bacterial synergism induced disease) and paraquat toxicity (a herbicide which when inhaled or ingested can lead to terminal pulmonary failure) will be examined in some detail.

LUNG IMMUNITY

The respiratory tract is exposed to a plethora of particles, gases, and microorganisms, and enclosed environments tend to concentrate such pollutants. Several defense mechanisms in the respiratory tract are involved in removing such pollutants. The nasus mucosa and turbinates filter out particles greater than 10 μ size and some soluble gases such as SO_2 go into solution on the moist mucosal surface; some smaller particles (down to 1 μ) are deposited on the mucus covered ciliated epithelial cells between the larynx and terminal bronchioles. Dust and small particles not trapped may be deposited at the bronchiole-alveolar junction which, because of aerodynamic factors, tends to cause particles to settle. These particles may eventually be cleared by the mucociliary escalator, although clearance from this deep anatomic location is very slow. Indeed, pathologically the lungs of elderly people and animals tend to have a lot of dust in this region of the lung parenchyma. This complex clearance mechanism begins in the respiratory bronchioles and ascends to the larynx. A reduced rate of mucociliary clearance has been associated with increased susceptibility to pulmonary infection (Ettinger 1985). Indeed, parainfluenza viral infection has been shown to impair mucociliary clearance and inflammatory cell function which may predispose to secondary bacterial infection. Normal mucociliary clearance requires morphologically and functionally intact ciliated epithelium and mucus with normal quality physicochemical and rheological properties. Exposure to airborne dust, allergens, irritants or microbes may reduce mucociliary clearance by altering the viscosity of the mucus layer or destroying mucus producing goblet cells. The critical components of this system include a sol/gel mucus system and the coordinated activity of thecliated epithelium, which moves the mucus blanket toward the larynx from which it is expelled by cough and then swallowed. This mechanism also provides a means of

dissemination of infectious, e.g., Mycobacterium tuberculosis, or parasitic, e.g., Dictyocaulus viviparus, agents to the gastrointestinal tract. Exogenous agents deposited in the proximal respiratory bronchioles, distal respiratory bronchioles, and alveoli are cleared by macrophages. Macrophages are cleared through the mucociliary escalator or may migrate to the bronchial associated lymphoid tissue for humoral or cellular mediated processing of the antigens. Some agents which are resistant to clearance via macrophage toxicity, e.g., asbestos particles, can set up a vicious cycle whereby a progressive inflammatory process can progress to pulmonary empyema. Asbestosis may eventually result in development of a mesothelioma. The normal pharyngeal flora, particularly the alpha hemolytic streptococci, are inhibitory to the growth of many potential respiratory pathogens such as Streptococcus pneumoniae, Staphylococcus aureus, and other gram-negative bacilli. Mucosal immunity, specifically secretory IgA, has direct microbicidal effects in addition to aiding in the recognition and phagocytosis of pathogens (Ettinger, 1985).

When the normal lung immune mechanisms are overwhelmed by an inoculum of bacteria, the lung cellular response consists primarily of segmented neutrophil infiltration. Other inflammatory responses also occur such as increased perfusion, capillary permeability and increases in interstitial and alveolar fluid. The alveoli quickly become filled with fibrin-containing fluid, bacteria, neutrophils, and erythrocytes. During infection with some rapidly multiplying pathogens, which resist phagocytosis, the early alveolar exudate provides a culture medium which may play a role in the spread of the bacteria through the lung parenchyma.

The normal lung immune status is a function of host factors (characteristics of Bronchial Associated Lymphoid Tissue (BALT) T/B ratios) and environmental factors (particulate or biological pollution). Expression of clinical disease depends on the interaction of these factors (Figure 1). For example, chronic exposure to ozone, a gas which results in free radical production, alters the pulmonary response to Listeria monocytogenes. Pulmonary oxidative stress alters delayed type hypersensitivity responses and lymphoproliferative responses in spleen and BALT to Listeria antigens (VanLoveren et al, 1988). The Listeria-induced pulmonary lesions consisting of multifocal infiltrates of histiocytic and lymphoid cells were intensified by prior oxidative stress.

PATHOPHYSIOLOGY OF PULMONARY INFLAMMATION

Surface tension forces in the fluid lining the alveoli account for the high pressures needed to inflate atelectatic lung regions. A

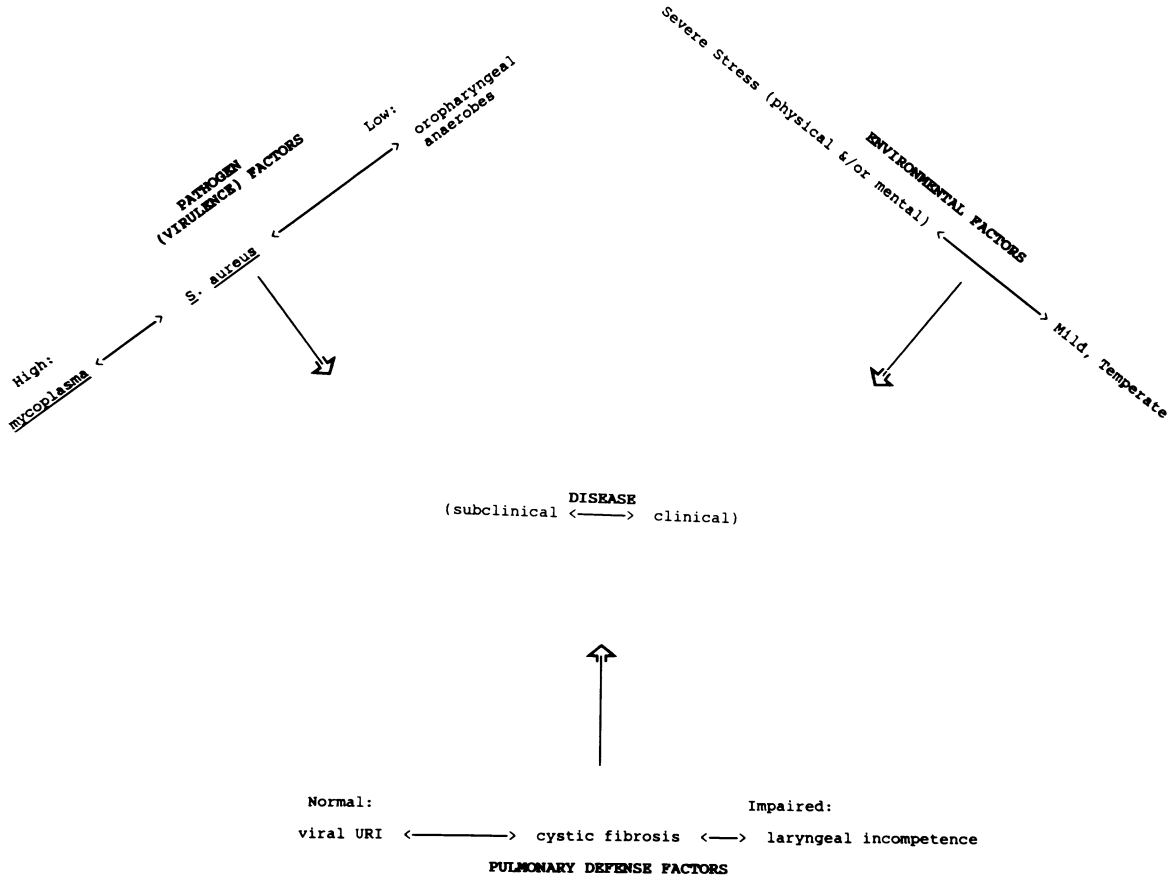

Figure 1. Relative Contributions of Disease Agent Virulence Factors and Host Pulmonary Defense Factors in Causing Pathology.

phospholipid surfactant produced in Type II pneumocytes acts to reduce surface tension in the alveoli which varies with lung volume. Measurement of lung compliance curves (dv/dp) can be used to differentiate obstructive versus restrictive disease. Decreases in lung dv/dp ("stiff" lungs) may result from exudates or interstitial edema, e.g., pneumonias, pneumonitis, or from diffuse interstitial pulmonary fibrosis, e.g., pneumoconiosis. Functional residual capacity (FRC) is defined as the resting end expiratory lung volume and is determined by the balance of inward lung recoil and outward recoil of the thoracic wall. Therefore decreased lung dv/dp reduces FRC and increased lung dv/dp increases FRC.

Restrictive lung diseases are a class of respiratory diseases in which lung inflation is limited by factors within the lung or thoracic cage. Restrictive lung diseases as caused by airborne agents include pulmonary fibrosis, interstitial edema, pneumonias or pleural effusion secondary to a pneumonia.

Obstructive respiratory diseases are a result of increased upper airway resistance which limits air-flow into or out of the gas-exchange areas of the lung. Since both bronchi and bronchioles possess smooth muscle in their walls, their diameter can be altered by a variety of airborne irritants or allergens. Exposure of the airways to irritants or allergens can result in mast cell histamine release which directly and indirectly (via a vagal reflex) causes airway constriction. In the alveoli, air-blood gas exchange occurs normally when blood has traversed half of the capillary, leaving some reserve for pathological conditions. Development of edema or exudates in the alveoli or a loss of alveolocapillary-surface area may impair oxygen diffusion and increase the diffusion component of the alveolar-arterial oxygen difference. Diffusion abnormalities affect the transfer of oxygen but not that of CO_2 which, due primarily to its greater solubility, is 21 times more diffusible between the blood and gas phases.

BOVINE PNEUMONIC PASTEURELLOSIS

The critical role of lung immunity and development of pathologic lesions is highlighted by the pathogenesis of bovine pneumonic pasteurellosis. The "shipping fever" complex (a severe fibrinopurulent necrotizing bronchopneumonia), is caused by Pasteurella haemolytica (serotype 1 ST1). Pasteurella haemolytica ST1 is part of the normal nasus flora in cattle and is present in low numbers in ambient air (Grey et al., 1971), but are not normal lung flora. In the process of shipping cattle to a feedlot, the number of P. haemolytica ST1 progressively

increases on the mucosa of the upper respiratory tract, tracheal air and eventually in the lungs (Frank and Smith, 1983). Exposure to parainfluenza-3 or infectious bovine rhinotracheitis virus will cause an increase in upper respiratory tract numbers of P. haemolytica (Frank et al., 1986). Preexisting infections may enhance susceptibility to pneumonic pasteurellosis by impairing the function of alveolar macrophages (Jakab, 1981). A P. haemolytica serotype 1 leukotoxin destroys alveolar macrophages and neutrophils at subtoxic concentrations impair chemotaxis (Markham et al., 1982) oxygen dependent activation of neutrophils both of which are important in bacterial clearance from the lung (Chong et al., 1985). Dying neutrophils in the alveoli stimulate inflammation and tissue necrosis as a result of lysosomal enzyme release. The leukotoxin is indirectly responsible for pneumonia and depletion of circulating neutrophils with hydroxyurea prevents pneumonic pasteurellosis (Slocombe et al., 1984).

OTHER CAUSES OF RESPIRATORY DISEASE

In cattle, IBR (Herpes virus) PI-3 and BRSV (paramyxoviridae) account for the majority of clinical respiratory diseases with rhinovirus, adenovirus, bovine viral diarrhea (non-cytopathic and immunotolerent forms of the disease), and malignant catarrhal fever contributing less than 1%. Common bacterial pathogens closely related to pasteurella include Actinobacillus lignieresi and Actinomyces bovis. Both are also known to cause tongue and bony lesions, respectively. Corynebacterium pyogenes can cause a suppurative pneumonia which often results in development of lung abscesses. Hemophilus somnus is often synergistic with or secondary to BRSV infection. Fusobacterium necrophorus is a common anaerobe which causes calf diphtheria, pyonecrotic lesions, and is often isolated in cases of a secondary septic pleuritis. The normal host for Mycobacterium bovis is cattle although it is infectious to man, cats, horses, and pigs by either ingestion or inhalation of droplet nuclei. Mycobacterium tuberculosis, the normal host of which is man or other primates, can be transmitted to cattle, pigs, and dogs. Clinically significant respiratory mycoses are usually limited to dogs, cats, and horses. A herpes virus causing feline viral rhinotracheitis (URT), caliciviral infection (LRT), and Chlamydia psittaci are the major clinically significant respiratory pathogens in cats. P. multocida (a different strain from that which infects large animals) is frequently a cause of feline pneumonia and secondary pleuritis (Table 1).

Cryptococcus neoformans, which is limited primarily to the

southwestern U.S.(Arizona and California), has been reported to cause nasal granulomas and sinusitis in cats. Airborne spores are inhaled and the organisms convert to the yeast form in tissues. This agent is infectious although not contagious. The disease may take one of three paths: (1) subacute infection eliminated by lung cell mediated immunity; (2) an acute disease resulting in granulomas in the lungs and tracheobronchial lymph nodes and other bronchial associated lymphatic tissue; and (3) a chronic disease characterized by granulomas and dissemination to other sites such as bone, liver, spleen, eyes and intestine.

INFLUENZA

Influenza is an acute, contagious disease following infection with certain myxoviruses. There have been five influenza pandemics during the present century. The viruses isolated during the pandemics of 1946, 1957, and 1968 have proved to be antigenically distinct with respect to their hemagglutin antigens. Indeed, the severity of pandemics can roughly be correlated with the degree of antigenic shift in hemagglutin or neuraminidase antigens. The biologically active peptides (hemagglutin and neuraminidase) are the primary determinants of immunity and mutate independently. This was clearly evident in the replacement of H2N2 by H3N3 (Hong Kong) virus in 1968. The hemagglutin is responsible for binding the virus to the cell. Antibodies to that protein will neutralize the virus. Viral neuraminidase plays a role in release of virus from cells; antineuraminidase antibody is not neutralizing, but limits viral replication and therefore the course of infection. As external spike projections from the viral envelope, H and N are in immediate contact with host antibody during infection. Therefore they are under selective pressure. When major antigenic shifts in the virus occur (perhaps as a reciprocal of high immunity in the general population), the acquired immunity in the population at risk may be inadequate to prevent infection and development of disease. The source of markedly different influenza A variants is not known with certainty, although some evidence suggests that recombination of human and animal influenza viruses may be occurring in nature. Indeed, the genome of the influenza virus is segmented which allows the virus to combine its gene segments with other viral strains. For example, swine influenza virus can combine with the avian strain to create a new flu virus.

PARAQUAT TOXICITY

Paraquat, a bipyridylium herbicide, is a potent pulmonary toxin

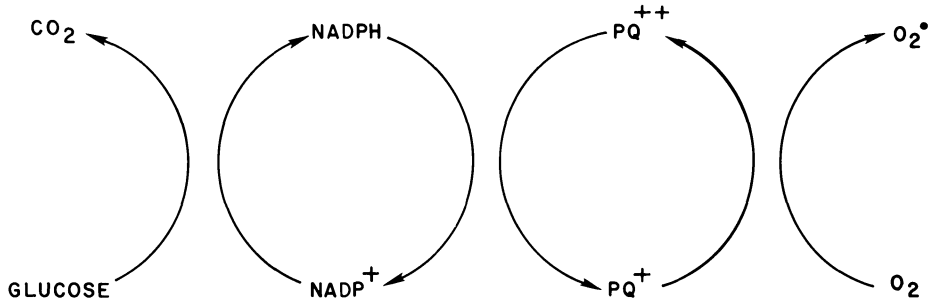

Figure 2. Pulmonary Oxidation of Paraquat and the Generation of Oxygen Free Radicals.

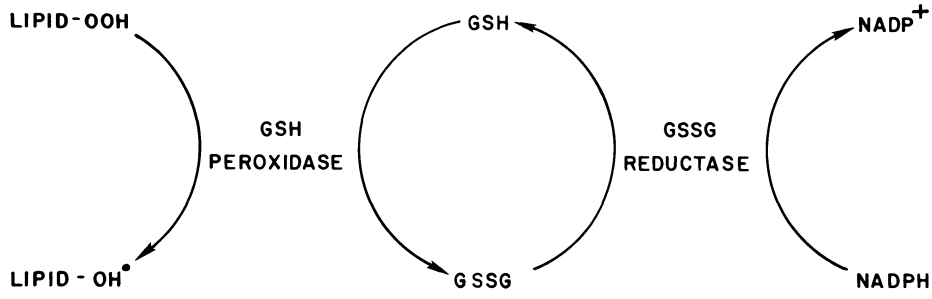

Figure 3. Role of Glutathione in Limiting Oxidative Stress.

following either inhalation, such as during spraying operations, or ingestion. Several years ago, tainted marijuana plants, apparently imported from Mexico, created a great deal of concern among marijuana users because of the potential for permanent and possibly fatal lung damage following use of the drug. The lung tends to concentrate Paraquat through an energy dependent mechanism. Histopathologically, paraquat toxicity causes several characteristic changes including thickened alveolar walls and cuboidal cells lining alveoli, and hemorrhage. Paraquat toxicity causes an increase in lung glutathione activities of glucose-6-phosphate dehydrogenase, and glutathione reductase indicating an oxidative stress and an increased demand on NADPH (Dunbar et al, 1988) (Figure 2). Furthermore, Paraquat induces an increase in lung polyamine synthesis. This may be involved in the repair mechanisms or may mediate the fibrotic changes following lung injury (Dunbar et al, 1988a). Oxidation of Paraquat in the lung results in the formation of oxygen free radicals which may damage tissue through lipid peroxidation, increased membrane permeability, enzyme inhibition, nucleic acid damage, cellular cytoskeleton disruption, and release of calcium stores from mitochondrial stores (Figure 3).

SUMMARY

The cost of pulmonary infections, in dollars and human life, has been growing astronomically in the past two decades. This review examines the deterioration of air quality by both biological and physical agents. It uses an example of each agent (bovine pneumonic pasteurellosis, paraquat toxicity) in attempting to show how the physical environment is changing and how biological agents evolve into new forms.

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SECTION IV

BIODETERIORATION OF CULTURAL PROPERTIES

BIODETERIORATION OF CULTURAL PROPERTIES

INTRODUCTION

Exploring the problems of biodeterioration of cultural artifacts and proposing solutions to those problems, are the goals of this session. Of the many degradative processes at work on an object, perhaps the most complex and least understood by conservation scientists, conservators, and restorers is biodeterioration.

The environmental circumstances conducive to microbial deterioration, either inside or outside a museum, need to be described in practical terms for those who are responsible for cultural properties. A major difficulty in the field of conservation is quantifying the effects of microbial attack relative to other forms of deterioration. Ultimately, of course, the main goal is to effect a remedy that is suitably benign to the object and the applicator, at the same time as it is sufficiently deadly to the microbes. It is desirable too that the treatment cure the problem for as long as possible.

The papers presented in this session make a significant contribution toward these goals. An overview of the field was provided in one paper that should serve as an introduction to the practical problems faced by conservators and conservation scientists who have to address these issues on a day to day basis. Included in this introduction were research suggestions that could help answer some of the questions in the field. In another paper, results from a specific conservation effort, that of removing biological growth from an outdoor Carrara marble, should serve to illustrate the current procedures followed by conservators of cultural properties made of stone. Also, apparent in this study is the type of interactive problem that may occur between chemicals used to remove microorganisms from stone, and, by extension, other media, and chemicals used to waterproof of consolidate stone. Introduced in this symposium as well were the issues surrounding the need to search for alternative strategies of long-term control for microbial growth. This last paper

discussed the use of an inert environment to reduce aerobic growths on important Egyptian mummies. This represents an innovative, and apparently benign, attempt in preventative conservation.

There is much work yet to be done in this field. It is my hope that the papers presented here, and in this series of symposia, will spark studies that elucidate the problems in conservation research.

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Biodeterioration in Museums--Observations

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INTRODUCTION

The understanding and awareness of the occurrence and importance of microbiologically caused deterioration within a museum context vary considerably from institute to institute within the art conservation community. While understanding the nature and severity of a microbiological problem is difficult in itself, perhaps even more difficult is prescribing a safe and effective treatment for an object of artistic of cultural value. This introduction will review some of the issues art conservators face when confronted with a microbiological problem. Suggestions are made towards bridging the gap between conservation and research in the field; in addition, suggestions on directions for future research are included.

Museums, such as The Metropolitan Museum of Art, in New York, that have the resources to completely enclose their collections within a carefully controlled environment often tend to ignore the concept of microbiological attack--until something goes wrong that requires a safe and effective treatment. Museums without temperature and humidity control are much more likely to be faced with the need to recognize and control microbiological attack on their objects, and are thus probably better prepared than those institutions able to control their internal environments.

The individual usually called upon to solve any museum object problem is the art conservator. For microbiological problems, this places the conservator in the unenviable position of having to recommend and undertake a treatment program that is effective in eradicating the problem while at the same time not altering the objects appearance or

informational content. The solutions are usually not simple as they require an understanding of the micro-organisms involved, the type of substrate they attack, the nature and rapidity of the attack, and the means of removing the organisms without affecting the material. Recognition of incipient microbiological attack and its safe removal is a subject rarely covered in detail in conservation program. This is an area where the research scientist can make an important contribution to the museum by providing the conservator with the information needed to understand when a microbiological problem is present, how deleterious it is likely to be and--most important--to suggest possible solutions.

One aspect of a successful collaboration between conservators and scientists is an understanding of the constraints under which conservation is carried out. Often a conservator is working under a time deadline, such as for installation of a show, and does not have the luxury of conducting a large amount of experimental research to fully understand the nature of the problem. On the other hand, for a scientist, it is often difficult to know the level of conservation science is--in terms of having adequately tested available conservation treatment strategies--when dealing with biological problems. The available literature in the field is generally scattered among numerous specialized conferences of varied utility and availability.

APPROACH TO CONSERVATION STUDIES/PROGRAMS

An all-encompassing approach to a biodeterioration problem requires identification of the problem, determination of the cause, correction of the source of the problem, and treatment of its effects. The following steps are required:

--Identify affected materials
--Identify the potential treatments
--Describe the potential treatments
--Perform preliminary screening/testing of best treatment choices
--Test response of test material
--Perform in situ testing
--Total object treatment

In many cases, problems in biodeterioration focus on a group of organisms, i.e., algae and fungi. In the latter group examples of experimental designs which incorporate elements of the above approach into conservation studies can be seen in the flow chart in Rebrikova (1984) and in Koestler and Santoro (1988). Unfortunately, many conservation treatment programs in existence provide little towards the understanding

of the organism or follow-up studies after arbitrary selection and implementation of a treatment program.

There is a strong need to update knowledge of those working on art materials. For example, Allsopp and Allsopp (1983) have produced an excellent survey list of commercial industrial biocide products available giving trade name, active ingredient, field of application, recommended concentration, countries where the product is available, the producer, and toxicity data, such as LD_{50}. A manual for fumigants used in the U.S. museum world has been compiled by Zycherman and Schrock (1988). This book-length manual includes relevant laws and public policy issues, pests and their identification, treatment strategies, pesticides, and hazards to personnel and material. The same kind of information is not available for biocides of utility to the conservation field.

Depending upon the object and prior handling, there is an essential need that proper records be kept for the object indicating handling, treatment, and coatings history. It is important to know all the chemicals in a treatment or biocide before use--often the chemical composition varies with lot number--and determine the probable effect of those chemicals on the material and on those who will handle the objects. In addition to the treatments or biocides, the solvent or carrier material should be known and checked and any adverse effects on the art object or for toxicity to the conservator.

Many of the above shortcoming contribute to confusion concerning conservation treatments. Many studies performed in the field are indicative but not definitive and lack basic information. Poor experimental design, preparation, in addition to poor data collection and/or analysis have been the norm of many studies performed to date--as pointed out in a controversial review by Reidy and Reidy (1988) of published studies in the conservation field.

Confusing information abounds also on the use of biocides. For example, manufacturers may encourage the use of their products, such as phenols (Paulus and Genth, 1983), without noting the special circumstances of art conservation. In the case of phenols, while some may be of utility to the field, others could encourage growth on certain materials or produce deleterious byproducts as a result of chemical interactions with the art object.

The use of fumigants in art conservation has progressed from an attitude of optimism to one of extreme caution. In the recent past for example, methyl bromide, ethylene oxide, or a mixture of the two were said to cause no harmful effects on art objects, and consequently were extensively used. However, subsequent research clearly demonstrated that

methyl bromide reacts with proteins to weaken them, and ethylene oxide stays in the materials especially in lipids, and proteins. In addition, reactions may occur between these chemicals and the object to produce byproducts that are deleterious to the art objects or to people (cf. Zycherman and Schrock, eds, 1988).

CONCLUSIONS AND SUGGESTIONS FOR FUTURE STUDIES

There are many new studies and programs that deserve discussion and research. Some of the most important biological issues in conservation are:

- --How to demonstrate the relative importance of microorganisms in the deterioration of materials as compared to other forms of object deterioration;
- --How to equate the biological component of deterioration with a meaningful abstraction, such as a "biodeterioration index" that can be compared to other types of deterioration, such as acid rain, water dissolution, or wind abrasion;
- --Development of new, or application of existing, biocidal control methods such that the art historical content is unaffected;
- --Assessment of the long-term effects of previous treatments on the objects and the people who handle them, e.g., ethylene oxide;
- --Conduct adequate follow-up studies on the effectiveness of the treatment. (In some cases a fungicide treatment actually encouraged growth after interacting with the material;)

Some specific efforts which should receive attention are noted below:

- --Follow-up studies are recommended for a series of treated stone monuments in Indonesia where epoxy and acrylic treatments were used in addition to biocidal agents to help preserve temples. This represents essentially a 15-year field trial.
- --Continued investigations on the effect of lichens on art objects. Should they be removed? If so, should the object be consolidated before of after removal?
- --Can bioluminescence of the ATP-ATPase system or flow micro-fluorimetry be used as a reliable method to derive a "biodeterioration index" of an art object?
- --Increased interest in the use of biocides necessitates that a biocidal treatment newsletter be setup. Perhaps under the auspices of the Pan-American Biodeterioration Society.

In conclusion, our hope is that more scientists, especially biologists, will become interested in the challenge of applying their expertise to help art conservators and conservation scientists define and solve the many biodeterioration problems that arise in the maintenance of museum collections and cultural and historical monuments.

SUMMARY

This paper introduced some of the important issues art conservators face when confronted with a microbiological problem. Suggestions were made towards bridging the gap between conservation and research in the field; in addition, suggestions on directions for future research were included.

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Nitrogen for Biodeterioration Control on Museum Collections

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INTRODUCTION

The conservation and preservation of biodeteriorated art materials is one of the most critical problems in museum collections. Recently, an increasing number of methods have been used to identify and eliminate biological contamination on valuable ancient objects (Polacheck, 1988).

Among the various methods used to prevent biological damage to artifact materials such as paper, parchment or wooden objects, fumigation has been employed most frequently for microbial control and insect eradication. However, fumigation is currently carried out using gases such as ethylene oxide or methyl bromide which involve risk of toxicity for museum personnel (Lewin, 1986), and have been found to produce chemical and physical alterations in the treated materials (Green, 1987). Studies show that fumigation can also increase the risk of damage when treating new contamination on museum materials previously sterilized with germicides (Kowalik, 1980).

Other alternatives have been explored by Nakamura et al. (1983), who used low oxygen levels for retarding microbial growth in stored food. Recent investigations (Valentin, 1989) have reported that low relative humidity and oxygen levels can reduce the biological activity of microorganisms and eradicate insect infestations in contaminated museum collections. In the present work, comparative analyses were carried out to determine the optimum relative humidity, temperature and oxygen concentration required to control bacterial, fungal, and insect infestation on art materials. An approach has been developed in which normal air was replaced by an inert gas, nitrogen, at low relative

humidity in a hermetically sealed case to retard the biological activity of fungi and bacteria on proteinaceous objects. The effect of nitrogen as anoxant on insect populations has been analyzed using Drosophilia melanogaster, as a model and has been used to eliminate insect infestation by termites, Cryptotermes brevis, in wood samples.

MATERIALS AND METHODS

New parchment was used for analyzing fungal activity under different conditions. Contaminated ancient parchment was employed for the evaluation of bacterial growth, while D. melanogaster and C. brevis were used for assessing the efficacy of nitrogen in eliminating insect activity.

Fungal Activity Evaluation Procedure

The effect of low oxygen and RH on inoculated parchment samples was assessed. A pure culture of Aspergillus flavus, considered resistant to low relative humidity (RH) (Zlochevskaya, 1986), was isolated from a proteinaceous art material and used as a model to determine the appropriate incubation time for the treatment of proteinic materials

In this way, following the method used by Valentin et al. (1989), three groups of 39 samples each were infected with a spore solution of A. flavus (20×10^4 spores/ml). Each sample was inoculated with 0.5 ml on either side. After one week of incubation, parchment samples with visible fungal contamination were hung from the stopper in 35 ml serum vials. Inoculated material was treated with a radioactive tracer, of a 14 µl (1:1) mixture of ^{14}C succinic acid, (0.32 mM) and acetic acid (0.36 mM), and exposed to various relative humidities (100%, 75%, 43%, and 33%). The air was then evacuated from the vials and mixtures of dry nitrogen and air at various concentrations were injected to achieve fixed oxygen levels: 20%, 1%, and 0.1%. Samples were incubated for 1, 2, and 3 weeks. The long term kinetics of labeled CO_2 produced by the microorganisms was measured by scintillation counting. This provide a quantitative measure of the overall biological activity under each condition. Silica gel was used to control the relative humidity in these experiments. Parchment samples exposed to random environmental contamination at 45% RH and 25C for 3 weeks were the controls.

Bacterial Growth Assessment

The analysis of viable and non-viable bacterial contaminants was

carried out using membrane filtration, a fluorescent dye, and epifluorescence microscopy. In this case, samples 15 mg each from old parchment were used as experimental material. Working with contaminated ancient parchment, an attempt was made to reproduce a real case of a material with possible aerobic and anaerobic bacterial contamination. As described above, three groups of 39 samples each, were attached to small hangers, hung from a stopper in vials, and respectively exposed to different relative humidities: 100%, 75%, 43%, and 33%, and various oxygen levels: 20%, 1%, and 0.1%. In this case, saturated salt solutions (Greenspan, 1977) were used to provide the range of RH inside the vials. The groups of treated samples were incubated for 3, 6 and 9 weeks at room temperature (20-22C). Samples exposed to ambient conditions (45% RH and 20-22C) for 9 weeks were used as controls. In this experiment a long incubation time was used to detect possible anaerobic contaminants.

After incubation, each sample was treated with 10 ml of PBS solution (Valentin, 1989), to obtain a microbial suspension which was filtered under vacuum through a polycarbonate membrane filter, pore size 0.6 μm (MacKinnon et al., 1981). The filter was stained with 5 ml of acridine orange 250 mg/l prepared in pH 6.7 phosphate buffer solution to stain the DNA of the bacterial cells retained by the filter (Denyer et al., 1983). The membrane filter was examined by incident UV light using a microscope with an epifluorescent illuminator system. A minimum of 10 fields of view were examined at random. In this way, the total number of bacterial cells was estimated.

The number of cells present in the total volume of liquid filtered represented the total contamination on the respective parchment samples.

Insect Eradication Method

The effect of nitrogen as an oxidant has been determined using D. melanogaster wild type for the experimental analyses, which permitted the assessment of a large population of each stage of the life cycle: eggs, larvae, pupae, and adults.

Cultures of D melanogaster were maintained in bottles containing a standard nutrient (Instant Drosopohila Medium, 4-24^{R}, Carolina Biological Supply Co., Burlington, NC). Each stage (eggs, larvae, pupae, or adults) was separated and placed into different bottles. In all cases 100 insects from each stage were evaluated under specific environmental conditions.

Groups of each stage of D. melanogaster were flushed for 30 minutes

with nitrogen using a flow rate of 200 cc/minute and various combinations of relative humidity and temperature: 75%, 40%, and 15% RH and 20, and 30C. The insects treated with nitrogen were incubated for increasing time periods between 10 and 100 hours (hr). A second set of insect groups in all stages was purged with air under identical conditions and used as controls. Each 10 hours of incubation, a set of treated Drosophila bottles was exposed to air at room conditions (22-25C and 45% RH). The mortality of eggs, larvae, pupae, and adults was then assessed after 20 days exposure to air. The humidity and the temperature of the gas were controlled using a thermostatic bath and a splitflow system air stream in which dry nitrogen was mixed with humidified nitrogen (Valentin et al., 1989). Temperature and RH inside the bottle were monitored by a Shinyei monitor. The oxygen content was measured using an oxygen analyzer (Teledyde). During the incubation periods the average oxygen concentration was 0.6%.

Based on this method, preliminary experiments were carried out using five pieces of wood (size 21 cm x 6 cm x 4 cm). The material was contaminated with 14 termites, C. brevis per sample. The contaminated wood was placed into different bags (100 cm x 75 cm) made out of plastiziced aluminum of low permeability, and purged with nitrogen at 200 cc/min. to replace oxygen by the inert gas at 40% RH and 25C for 8 hours. After exposure to nitrogen, each piece of wood was incubated for 15 days at various oxygen concentrations (20%, 3%, 2.5%, 1%, and 0.5%) and identical room conditions. After incubation, the wood was reexposed to ambient air for one week and cut to assess the mortality of the insects. Disinfestation treatments using large populations of insect pest museums and plastiziced bags of various permeabilities are being tested.

RESULTS AND DISCUSSION

Fungal Activity on Parchment Samples

The effect of low oxygen concentration and RH on fungal activity in parchment samples was analyzed. The results obtained indicated a significant decrease of fungal activity at 43% RH, 33% RH, and oxygen levels lower than 5% compared to samples exposed to high RH (100%) and ambient air (Figure 1). It was found that 3 weeks incubation time at low oxygen levels produced a decrease in the activity of A. flavus in all the samples analyzed at each RH including contaminated samples exposed to high RH (75% and 100%).

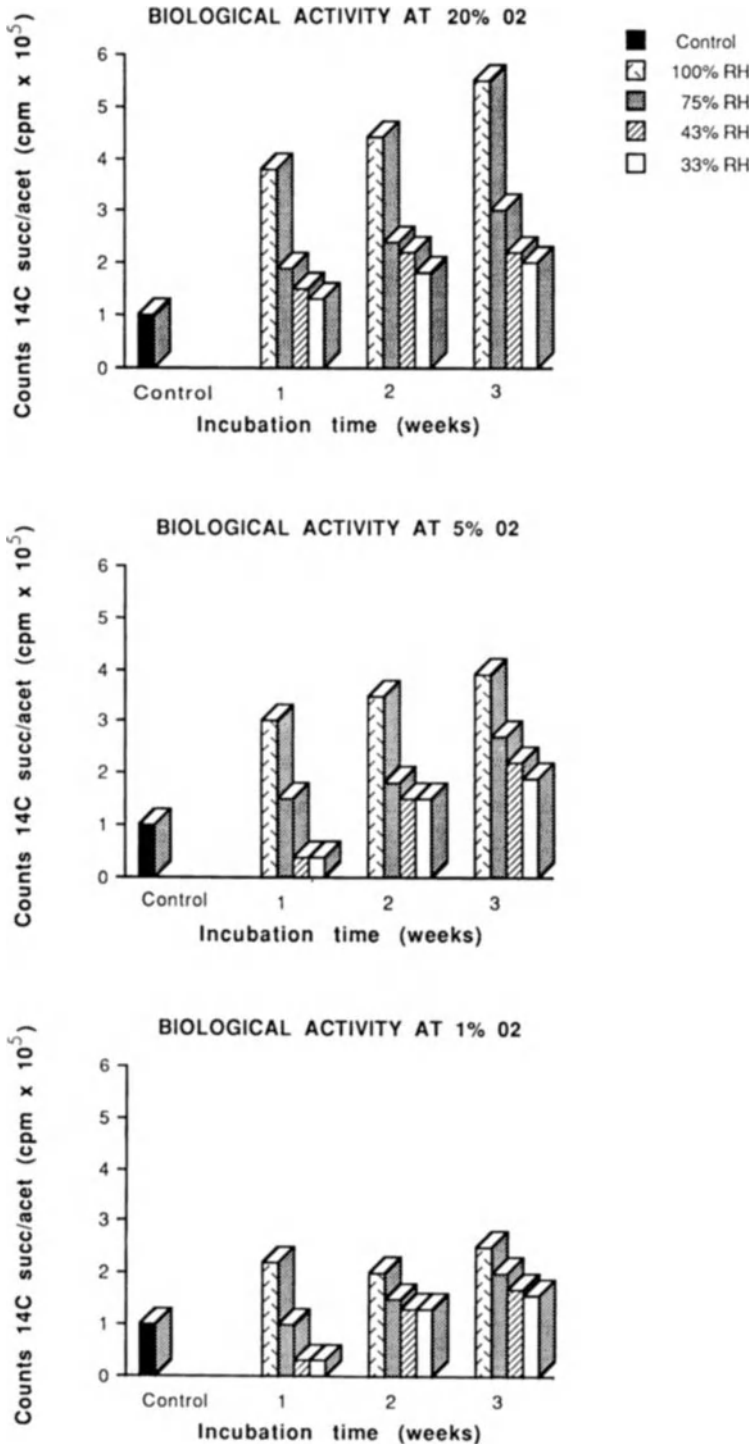

Figure 1. Biological Activity of Aspergillus flavus Inoculated on Parchment Samples and Exposed to Various Oxygen Concentrations and Humidity.

Experimental data (Valentin et al., 1989) showed that mixed populations of fungi and bacteria from random environmental contamination on parchment incubated at low RH and oxygen concentration for 36 hours presented a retardation of the biological activity after 10 hours of incubation reaching an equilibrium which indicates that inhibition of microbial activity had been achieved. It was also found that the incubation period necessary to retard the microbial activity can be diminished by decreasing the RH under 40% and the oxygen level under 1%. In this work, using pure cultures of A. flavus resistant to low RH, it was noted that more than three weeks incubation at 1% RH in the range 43-30% were necessary to achieve total inhibition of the microbial activity.

According to the results shown in Figure 1, it was also found that purging with dry oxygen-free nitrogen could be an effective treatment for water-damaged materials. It has been observed that when nitrogen is flushed through a display case containing wet materials, two effects are achieved: First, the biological activity of the aerobic microorganisms on treated objects is reduced due to oxygen exclusion; and second, the water content of the object is progressively decreased due to the effect of the replacement of the gas phase in the case. Using flow meters, the decrease in relative humidity can be controlled, and consequently flooded materials can be dried in a relatively short period of time at controlled relative humidity. Using this method for water-damaged materials, it is possible to simultaneously dry objects, decrease biological activity, and also reduce the physical deformations that are typically produced during drying processes employing normal air, especially in the case of proteinaceous art materials.

A sensitive experimental technique has been developed using radioactive tracers to evaluate the microbial activity on contaminated materials exposed to modified atmospheres. Other applications of radioactive tracers in the field of microbial contamination could be developed. For example ^{14}C tracer could be used for evaluating the growth of organisms such as sulfooxidizing and nitrifying bacteria, algae, or lichens, which are difficult to maintain as pure cultures. The degree of contamination of any organic or inorganic material could be assessed using the appropriate radioactive tracers, which would allow the measurement of microbial activity on a support.

Bacterial Growth on Proteinaceous Samples

Bacterial development on proteinaceous materials under specific

conditions was examined for the treatment of contaminated art objects. Figure 2 shows the number of bacterial cells on parchment samples exposed to different relative humidities and oxygen levels. In this experiment, a stable number of bacterial cells was found on all samples exposed for nine weeks to low RH 43%-33% at each oxygen concentration of incubation. In contrast, a significant increase of bacterial cells was found at 1% oxygen concentration and 75% RH after three weeks incubation as compared to all the samples tested. This fact could be interpreted because of the presence of anaerobic bacteria which need high humidity levels for their biological activity. We also found that the growth of bacteria on samples exposed to a nitrogen atmosphere (oxygen concentration 0.1%) and high RH (75%-100%), was lower compared to bacteria under 1% oxygen level at identical RH. Schegel (1986) has reported that anaerobic bacteria are influenced largely by the RH and the presence of CO_2. For this reason, a nitrogen atmosphere (with exclusion of oxygen and CO_2) and low RH will retard the development of bacterial growth including anaerobes.

Preliminary experiments using plate colony counts and anaerobic atmospheres (BBL Gas Pack Pouch) confirmed the presence of anaerobes on proteinaceous materials exposed to RH over 75% and oxygen concentration around 1% (Valentin et al., 1989). This result agrees with the data of Kowalik (1980) who found that ancient parchment and leather under high RH presented high contamination by anaerobic bacteria. Comparing both methods of analysis, we noted that the direct epifluorescent filter technique (DEFT) was the most sensitive means to evaluate bacterial growth. Moreover, it was found that using DEFT by staining the DNA of microbial cells it was easy to distinguish between microbial cells and other particles present in the samples analyzed.

In the present work, all the analyses have been carried out at room temperature, because most of fungi and bacteria have their maximal development rates at temperatures in the range of 20-42C. With respect to this parameter, our results showed that the combination of low relative humidity and low oxygen levels was sufficient to control the microbial activity at room temperature (approximately 20-22C).

Using radioactive tracers and membrane filtration and epifluorescent microscopy, we developed experimental approaches to the quantitative measurement of the overall biological activity and total microbial growth in a sample avoiding traditional plate counting procedures, which are not sensitive enough to evaluate the degree of contamination of a material because of the selective culture media.

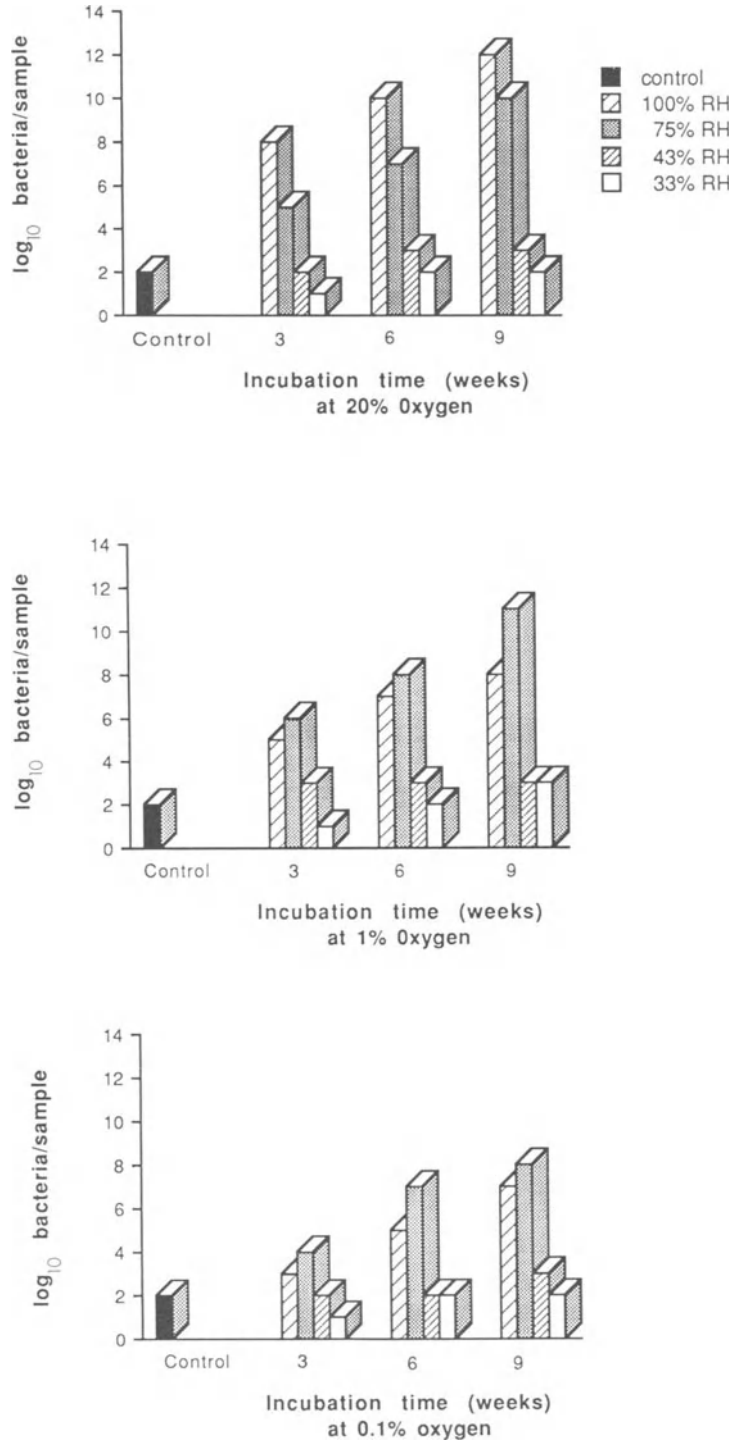

Figure 2. Bacterial Growth on Parchments Samples at Fixed Oxygen Concentrations and Relative Humidities.

Insect Control

The lethal effect of nitrogen as oxidant on all the stages of the life cycle of D. melanogaster was analyzed. Figure 3 exhibits the mortality in populations of adults, larvae, pupae, and eggs treated with nitrogen at different RH and temperatures, compared to control populations purged with air at identical conditions.

It was found that oxygen concentrations lower that 1% were effective in eliminating 100% of all the stages of D. melanogaster in a relatively short period of time: 30 hrs for adults and larvae, 60 hrs for pupae and 80 hrs for eggs. It was noted that the combination of low RH and high temperature had a drastic influence on the mortality of the insects, increasing the lethal effect of the gas. It was also found that below 1% oxygen, the combination of low RH (40%) and high temperature (30C) was effective for eliminating 100% of Drosophila in 30 hours, including eggs. Figure 3 shows that larvae and adults were the most sensitive stages to the effect of nitrogen as anoxant, while pupae and eggs were relatively more resistant.

The data obtained using a nitrogen treatment are dependent on: a) the cc/min of nitrogen flushed. b) The combination of low RH and high temperature which reduced the incubation time, increasing the mortality at all the stages of the life cycle. c) The age of the insects tested, especially for larvae pupae and eggs.

The potential application of this method for eradicating insects in museums was carried out using different pieces of wood which were contaminated by termites, C. brevis. Preliminary results shown in Figure 4 indicated that after 15 days of oxygen exclusion with exposure to oxygen concentration lower than 1% at 40% RH and room temperature 22°C, 100% mortality was achieved. Over 2.5% oxygen concentration the effect of nitrogen was significantly reduced.

CONCLUSIONS

It was found that a significant decrease of the fungal activity occurred on inoculated parchment samples exposed to decreasing relative humidities (43-33%) and low oxygen concentrations (1%) during three weeks of incubation.

No increase of bacterial cells was found on parchment samples exposed to 43-33% RH at each oxygen concentration tested during nine weeks of incubation compared to samples exposed to high RH at identical oxygen levels.

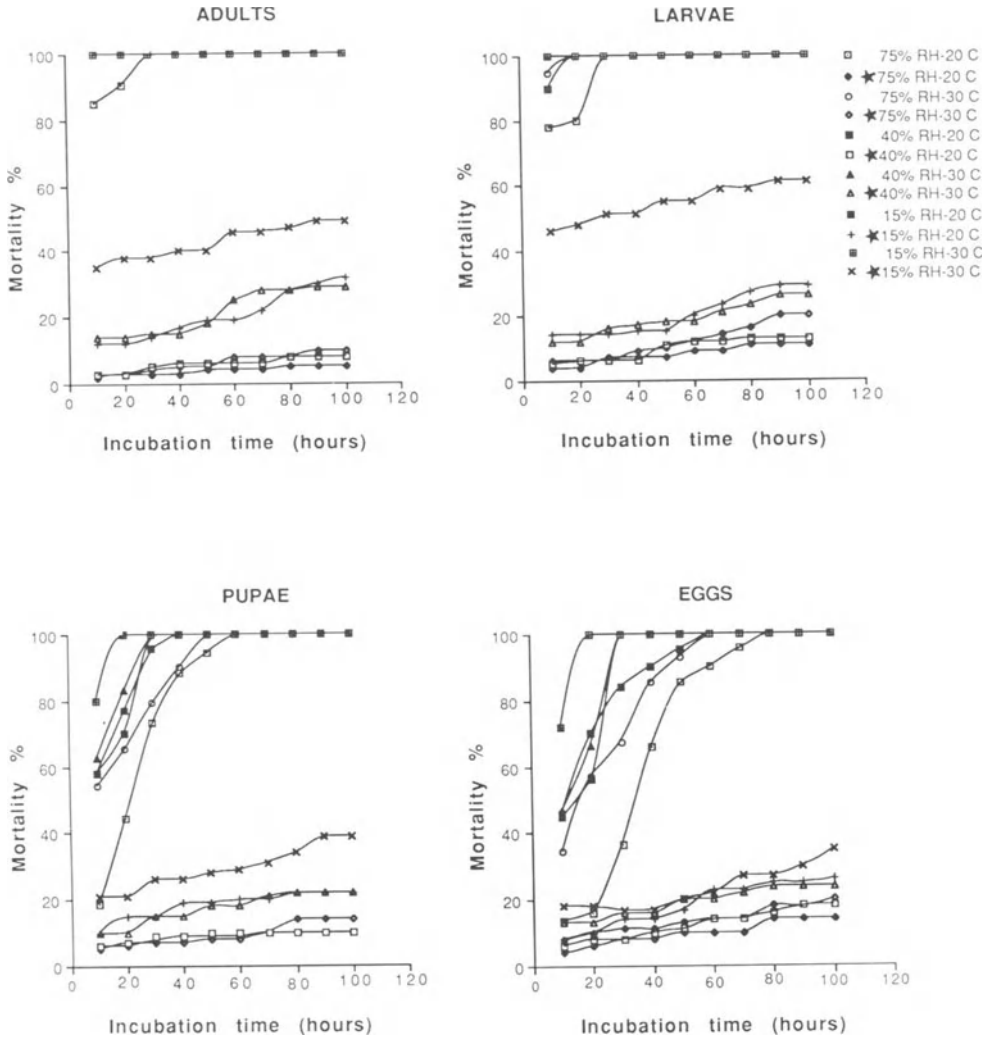

Figure 3. Mortality of All the Stages of Drosophila melanogaster Treated With Nitrogen at Different Relative Humidities and Temperatures, and Incubated at Various Times. (*) control population of D. melanogaster purged with air is so designated.

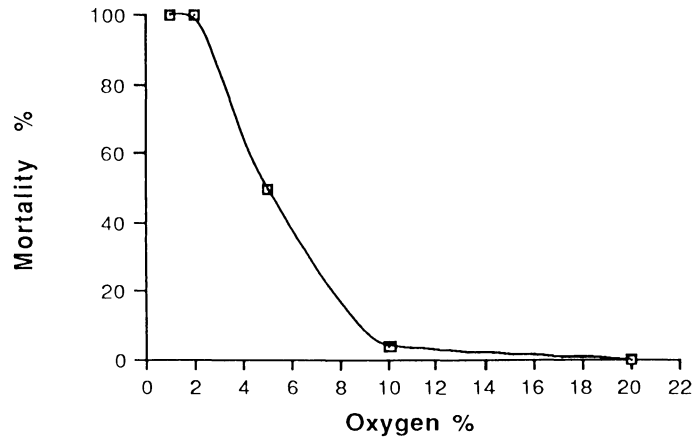

Figure 4. Mortality of Cryptotermes brevis on Wood Samples Treated With Nitrogen at Different Oxygen Concentrations, 40% RH, 22C for 15 Days Incubation Time.

It was also found that a nitrogen atmosphere was an effective method for controlling all the stages of the life cycle of D. melanogaster used as a model insect. D. melanogaster was a very sensitive insect to assess the effect of modified atmospheres on each stage of the life cycle, at various environmental conditions. Moreover, using Drosophila as a model, it was shown that a nitrogen atmosphere (below 1% oxygen concentration) during 15 days is an effective method of eliminating 100% of the C. brevis found in contaminated pieces of wood.

According to our experimental results and considering that art materials should not be exposed to extreme conditions, it can be suggested that low oxygen concentration (in the ranges 0.5-1%) 40%-45% RH, and 22-25°C, for 3-4 weeks incubation could be an effective means for the inhibition of microbial growth and eradication of insect pests from art objects of a moderate size.

Inert gases such as nitrogen are non-toxic, inexpensive, and easy to apply. Indeed, by using nitrogen, chemical alterations in the materials will be avoided. Consequently, this treatment could be an effective alternative to toxic gas fumigations in museum collections.

SUMMARY

A method of microbial control and insect eradication has been developed using inert gases, nitrogen, and low relative humidity in a hermetically sealed case. Radioactive tracers were used to assess the biological activity of fungal contaminants inoculated on new parchment samples and exposed to a nitrogen atmosphere at fixed relative

humidities. Bacterial growth was evaluated by membrane filtration and epifluorescence microscopy using ancient parchment under different levels of relative humidity and oxygen. The lethal effect of nitrogen as anoxant for eliminating insect populations was determined using D. melanogaster as an experimental model. A significant decrease of the biological activity and bacterial growth was found on contaminated material exposed to low levels of relative humidities and oxygen concentrations. Using D. melanogaster as a model it was found that a nitrogen atmosphere under controlled temperature and relative humidity is an effective means for eradicating all the stages of insect life cycle including eggs. Similar results were obtained using termites, C. brevis, an insect found in museums.

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A Case Study of the Compatibility of Biocidal Cleaning and Consolidation in the Restoration of a Marble Statue

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INTRODUCTION

The two major components of the restoration of an 1884 outdoor marble sculpture in New Orleans were cleaning the extensive biological growth and consolidating the fragile surface. New Orleans' semi-tropical climate encourages the growth of microorganisms that can be damaging to the stone. The biological growth on the sculpture of Margaret Haughery, a philanthropist (Figures 1a and 1b), was found to include algae, fungi, hemi-lichens and lichens, particularly Caloplaca feracissima H. Magn., which greatly altered the white Carrara marble surface appearance. In addition, some organisms had penetrated up to 10 mm into the stone along larger cracks (Figure 2) and 0.05 mm beneath loose surface crystals. Their physical presence contributed to the mechanical degradation of the marble. They also contributed to the stone's chemical degradation by etching the marble crystals with acidic secretions (Figure 3).

MATERIALS AND METHODS

After reviewing the conservation literature and conducting tests both on-site and in the laboratory (Figures 4 and 5), calcium hypochlorite $Ca(OCl)_2$ was selected as the biocidal treatment (Ashurst,

Figures 1a and 1b. The Statue of Margaret Haughery Before Treatment (Left) and After Treatment (Right).

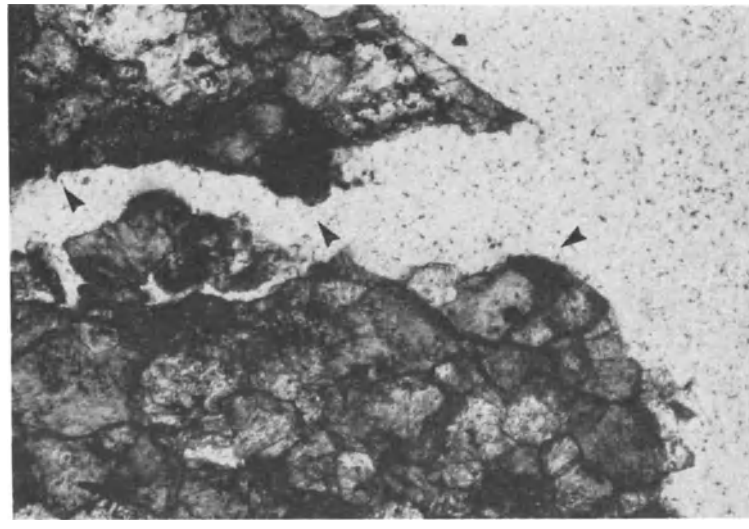

Figure 2. Thin Section of the Mouth of a Crack in the Marble 10 mm Deep (Approximately 2 mm Are Shown Here). Seen in normal transmitted light at 40x. Dark masses along the lining of the crack and beneath loose crystals are biological growth.

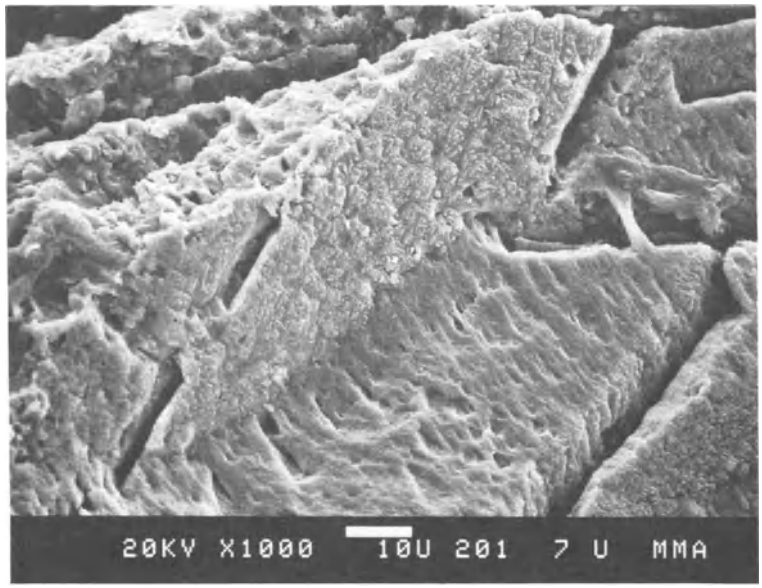

Figure 3. Scanning Electron Micrograph of Marble Crystals Showing Biological Growth Next to Etched Surface.

Figure 4. Laboratory Test of Seven Biocidal Cleaning Treatments on a Marble Tombstone Covered with Microorganisms. 1) water and detergent, 2) 30% hydrogen peroxide, 3) 0.75% calcium hypochlorite, 4) 1.5% calcium hypochlorite, 5) 3.0% calcium hypochlorite, 6) calcium hypochlorite grains, 7) Organic Stain Cleanser (Barov, 1987), and 8) a spray of 1% benzalkonium chloride. A clay poultice was used with 2 - 6.

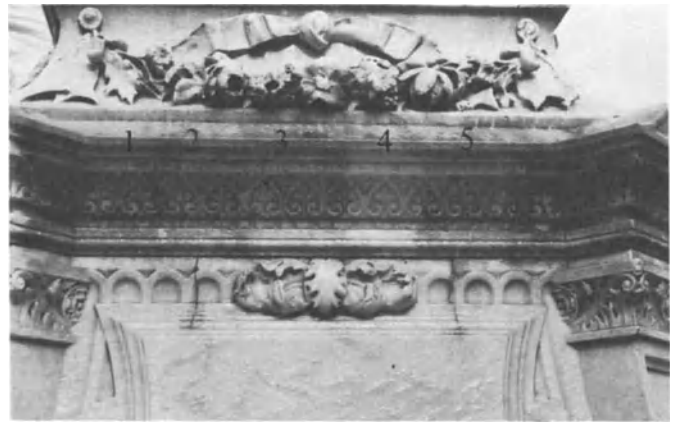

Figure 5. Results of On-Site Tests of Biocidal and Cleaning Treatments. 1) water and detergent, 2) Sure Klean T-1095 Special Liquid Marble Cleaner^{R} by ProSoCo, 3) Sure Klean Marble Poultice and Marble Poultice Additive^{R} by ProSoCo, 4) calcium hypochlorite grains and clay (less than 10% in solution), and 5) 30% hydrogen peroxide solution and clay.

1988; Barov, 1987; Fry, 1985; Grant and Bravery, 1985; Hale, 1978; Richardson, 1975; and Riederer, 1981 and 1986).

A commercially available calcium hypochlorite product was chosen for the field application, the HTH Dry Chlorinator^{R} from Olin Chemicals. It has the same percentage of available chlorine as the reagent-grade product (65%). The granular powder was mixed with equal parts of an inert clay, in this case talc, then filtered water was added to achieve the desired consistency. The treatment was first applied as a thin slurry, left for two hours then thoroughly rinsed with pressurized water spray. Areas of stubborn staining were treated with a thicker poultice which was left overnight before rinsing. Scrubbing with a nylon-bristled brush completed the removal of larger, tenacious growths on the surface. Good results were obtained on even the most darkly stained carved areas (Figure 6).

Recent investigations of the resistance of numerous consolidants to biological attack (Koestler and Santoro, 1988) noted Conservare H40 to have poor resistance to such attack. This prompted the selection of a new ethyl silicate product, H40 Plus^{R} by ProSoCo, that incorporated a biocidal agent into the consolidant. Although not tested in the above study, this additive was presumed to provide a measure of prevention for the consumption of the consolidant by microorganisms. Energy dispersive

x-ray spectrometry (EDS) revealed that the biocidal additive was an iodide.

The consolidant was applied using a stainless steel sprayer, in three cycles. The spray tip was held about three inches from the surface of the stone, and was moved at a rate that allowed complete absorption but no runs or drips. The three applications took about two hours to complete. According to the manufacturer's instructions, a rinse of methyl ethyl ketone was applied after one-half hour to an hour after the final spray cycle, to remove excess surface consolidant, which could lead to a glossy appearance.

Figure 6. Deeply Carved Area of Sculpture After Biocidal Cleaning Treatments. Refer to Figure 5 for comparison.

RESULTS AND DISCUSSION

An unusual yellow discoloration had appeared by the final spray application of the consolidant, particularly on thin or raised areas where evaporation occurred most quickly. The solvent rinse removed the discoloration except in deeply carved areas where consolidant and solvent were able to pool. This residual transparent yellow film was easily removed from those areas within twenty-four hours.

Subsequent laboratory tests discovered that the H40 Plus consolidant displayed slight discoloration when combined with even small amounts of calcium hypochlorite (i.e. 1/35 ml of a 1.5% calcium hypochlorite solution in 10 ml consolidant). No discoloration occurred when the H40 consolidant without the added biocide was combined with calcium hypochlorite.

Presumably, the biocidal iodide added to the H40 Plus reacted with the calcium hypochlorite solution. Two compounds associated with a yellow coloration that might possibly be the result of such a combination are calcium iodide (CaI_2) and iodine trichloride (Cl_3I). Further testing is needed to determine the exact nature of the reaction.

When the H40 Plus was combined with calcium hypochlorite crystals and allowed to solidify, the white crystals were surrounded by a bright yellow film. This film, along with a slightly yellow film of the H40 Plus and a drop of 3% calcium hypochlorite solution, was analyzed by energy dispersive x-ray spectrometry (EDS). The results are given in Table 1.

As noted in Table 1, Sample #1 shows that a solidified film of H40 Plus alone consists mainly of silicon, with tin as a catalyst for the consolidation reaction and a small amount of iodine, presumable as the biocide. Sample #2 was from the regular H40 (no added biocide) with a drop of 3% calcium hypochlorite solution; only silicon and tin were present. Sample #3 is the H40 Plus with a drop of the 3% solution. The elements detected were silicon, tin, iodine, chlorine and sulfur. No calcium showed up in this sample. Sample #4 was taken from the white area that formed right around the calcium hypochlorite granules when they were combined with the H40 Plus. The elements detected were silicon, sulfur, chlorine, calcium, tin and iodine. Sample #5 was taken from the yellow film that formed between the white areas when the calcium hypochlorite granules were combined with the H40 Plus. The same elements were found as in Sample #4, but the calium and chlorine were proportionally lower and the iodine higher than in #4.

SUMMARY

These tests provided some interesting information. They seem to indicate that the iodine added as the biocide reacts with either calcium or chloride ions to cause the yellow coloration. It is not known if the iodine reaction renders the product less effective as a biocide or as a consolidant. Experiments to test that assumption are planned.

The identification of the trace amounts of residue left on marble after poulticing with calcium hypochlorite grains and water rinsing is also important. It seems likely that it might be calcium chloride, which is a salt that is twice as soluble in water (74g/100g H_2O) as sodium chloride (35.7g/100g H_2O). If so, prolonged water rinsing or natural weathering should remove the residue fairly quickly.

Table 1. Results of Energy Dispersive X-ray Spectrometry Of H40 Plus and Calcium Hypochlorite Crystals[a].

Sample[b]	Element[c]	Relative Weight %	Relative Atomic %
#1	Si	95.3	98.8
H40 Plus	Sn	3.0	0.7
	I	1.5	0.3
#2	Si	97.6	99.4
H40 with	Sn	2.3	0.5
3% cal hypo sol.			
#3	Si	91.7	97.1
	S	0.9	0.8
H40 Plus	Cl	0.3	0.2
with 3% cal hypo	Sn	4.0	1.0
solution	I	2.9	0.7
#4	Si	67.3	75.8
	S	1.4	1.3
H40 Plus	Cl	6.8	6.1
with cal hypo	Ca	19.5	15.4
crystals	Sn	3.2	0.8
white area	I	1.5	0.3
#5	Si	78.4	86.5
	S	1.7	1.6
H40 Plus	Cl	4.9	4.3
with cal hypo	Ca	7.0	5.4
crystals	Sn	3.4	0.8
yellow area	I	4.3	1.0

[a]Accelerating voltage, 20.0 KeV; Beam - sample incidence angle, 60.0 degrees; x-ray emergence angle, 40.8 degrees; x-ray - window incidence angle, 8.3 degrees; window thickness, 20.0 microns.

[b]Samples consisted of 10 ml of consolidant with 1 drop (approximately 1/35 ml) of calcium hypochlorite solution. They were prepared by mounting them on spectroscopically pure carbon stubs with carbon paint, coating them in a vacuum evaporator with pure carbon and then with pure gold.

[c]This method of analysis detects elements having an atomic number greater than or equal to 11. Therefore the component analysis presented here will be different than chemical analysis.

One immediate question to conservators will be the sensitivity of biocidal consolidants to any foreign inorganic substances in the stone left either by previous treatments or environmental factors. Will these reactions jeopardize the consolidating ability of the products or their visual appearance?

Further research into the use of biocides on stone, their incorporation into consolidation treatments, and the use of these products in combination with other conservation treatments such as cleaning, has certainly been indicated by this project. Much experimentation and product testing is still needed to determine the most effective and least damaging combinations of biocides and consolidants.

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SECTION V

GENERAL BIODETERIORATION AND DEGRADATION

GENERAL BIODETERIORATION AND DEGRADATION

INTRODUCTION

Papers on a variety of topics have been grouped under this general heading. The increasing use of sophisticated instrumental methods to explore biodeterioration and related problems is clearly a timely subject. Several papers in this section deal with the use of instrumental methods to explore changes and deterioration at the molecular level. Nuclear magnetic resonance spectroscopy (NMR) was used to explore the binding of tryptophan to hemoglobin. Spin-labeled normal and sickled hemoglobin were allowed to bind fluorine-labeled tryptophan and the proximity of the tryptophan binding site to the nitroxide spin labeled was determined through the broadening of the ^{19}F signal. The enzyme monoamine oxidase, the most important enzyme involved in the degradation of biogenic amines, was the subject of a similar binding study, in which liver monoamine oxidase was reacted with spin-labeled tryptamine. Electron spin resonance (ESR) was used to monitor the line broadening effects produced by the binding of the spin-labeled substrate to the enzyme; the ESR spectrum was used to determine the dissociation constant of the substrate-enzyme complex and the approximate number of substrate molecules bound by the dimeric enzyme. The active site of monoamine oxidase was also explored through the use of fluorescence spectroscopy; the effect of monoamine oxidase substrates and fluorescence inhibitors on the fluorescence of probes bound to sulfhydral groups in the enzyme indicated that these groups are a part of the enzyme's active site.

The biodeterioration of textile fibers has been the subject of papers published in earlier volumes of this series. The advent of Fourier transform infrared spectrometers (FTIR) and infrared microscopes permit the quantitative infrared analysis of minute samples. This combination of techniques was applied to buried rayon and cellulose

acetate fibers and in the case of the cellulose acetate fibers, revealed changes in polymer structures that could be correlated to the period of burial.

Several papers examined biodeterioration in aqueous environments. The contamination of coastal waters by various Vibrio-species has been reported frequently in recent years. These Vibrio-species have been found in association with chitin-bearing organisms. The stimulation of Vibrio growth by chitin was explored in vitro in an attempt to identify factors that promote growth; the salinity of the aqueous environment had the greatest effect on growth, while temperature, pH and source of chitin were found to exert little influence.

The improvement of the treatment of aqueous waste is discussed also. The use of a microbial ecosystem to remove highly toxic metals from waste waters was successfully demonstrated. This unusual approach to detoxification has potential applications in the decontamination of waste water and in the recovery and recycling of heavy metals. The effects of the addition of microbial nutrients to waste stabilization ponds on the reduction of organic carbon were explored, using bis(2-chloroethyl)ether and 2-ethoxyethanol as test compounds.

Another group of papers in this section explore factors influencing the growth of microorganisms. The effects of temperature, nutrients and spore concentration on the germination of conidia of Dactylomyces thermophilus were extensively examined. The effects of aspartame and saccharin on the growth of a variety of microorganisms were determined.

The remaining papers measure biochemical responses. Protein profiles and protein deterioration in soybean cells that were stressed is discussed. Myodeterioration of muscle tissue due to serotonin toxicity is reported. Finally, changes and deterioration of surface ultrastructure by an antiparasitic phamaceutical is evaluated.

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Magnetic Resonance Investigation of Hemoglobin from Sickle Deteriorated Erythrocytes

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INTRODUCTION

Sickle hemoglobin under physiological conditions and concentrations aggregates upon deoxygenation to form a viscous gel composed of long fibers consisting of filaments of stacked rings, of which several detailed structures have been proposed (Finch et al., 1973). The important change in sickle hemoglobin is substitution of a non-polar hydrophobic residue (valine) for a polar residue (glutamic acid). This suggests that hydrophobic interactions are important in stabilizing HbS aggregation (Votano et al., 1977). A variety of small molecules containing hydrophobic moieties have been shown to inhibit the aggregation or gelation of deoxygenated HbS (Novak et al. 1978, 1979). These agents include aryalalkanes (Ross and Subramanian, 1977), the aromatic amino acids (Noguchi and Schechter, 1977, 1978), aliphatic alchols, amides and ureas (Poillon, 1980), a variety of oligopeptides (Kubota and Yang, 1977; Votano et al., 1977; Gorecki et al., 1980), and a variety of phenyl derivatives (Gorecki et al., 1980; Poillon, 1982). The inhibitory effects of these agents are believed to be due to a competitive interference mechanism in which the inhibitor binds to the HbS molecule at one of the important contact sites, and blocks other HbS molecules from binding at that site (Abraham et al., 1975; Behe and Englander, 1979).

The dynamic events that occur when inhibitors bind to proteins have recently become amenable to experimental attack by magnetic resonance techniques which depend upoon adiabatic half passage studies (Skyes, 1978), or broadening of the observed resonance. An approach which has been used to obtain simple NMR spectra for large proteins is to label the protein with a nucleus which is either not naturally occuring or not in high natural abundance, Fluorine 19 is a particularly attractive nucleus

from a NMR point of view because of the high sensitivity of ^{19}F NMR, the fact that ^{19}F is 100% naturally abundant, and the sensitivity of ^{19}F chemical shifts to the environment. Considerable success has been obtained in both the labeling of proteins with fluorine containing reagents and (i) the biosynthethic incorporation of fluoroamino acids into proteins and enzymes (Sykes and Weiner, 1980, and (ii) in the use of ^{19}F NMR to study these proteins and enzymes under a wide variety of conditions including incorporation into large membrane bilayers.

^{19}F-NMR spectroscopy represents a sensitive method for the investigation of molecular interactions involving a paramagnetic center. In particular, ^{19}F-NMR spectroscopy can be used to observe changes associated with the separate sites of a molecule interacting with a binding in proximity to a paramagnetic center. Such data should allow evaluation of the nature of the interaction or binding, accessibility to the binding site, molecular orientation, and potentially, the interpretation of the structural details of the binding caviety in both a structural and dynamic sense.

In this paper, is reported the use of ^{19}F nuclear magnetic resonance studies of the interaction of small hydrophobic molecules with normal and sickle hemoglobins. Also the use of spin label enhanced NMR relaxation as an approach to locating and characterizing the antisickling inhibitor binding sites is included.

MATERIALS AND METHODS

Materials

Blood from normal adults and from homozygous sickle patients was drawn into EDTA anticoagulant. The spin-labeled [(1-oxyl-2,2,6,6-tetramethyl)-4-piperidenyl)] [^{2}H, ^{15}N] maleimide (MSL) was a generous gift of Dr. Venkataramau at Vanderbilt University. The ^{19}F-DL-tryptophan, and ^{19}F-pyruvate were purchased from Sigma Chemical Co (St. Louis, MO). The other common reagents were of high quality. Solutions of cell-free hemoglobin were prepared according to Abraham et al. (1975) and maintained under N_2 atmosphere in the cold for 3 h. Electrophoresis of HbS solution showed the composition to be 95% HbS and 5% fetal Hb.

Methods

Hb and HbS samples were spin-labeled with nitroxide spin label [^{2}H,^{15}N] (MSL) following a previously published procedure (Zeidan, 1988). (Labeled Hb and HbS samples are noted as Hb^{+} and HbS^{+}, respectively). Water was exchanged with D_2O at pH 7.0. All solutions were treated with Chelex 100 (Bio-Rad) to remove residual paramagnetic ions.

Electron paramagnetic resonance (EPR) measurements were recorded at room temperature by a Varian EPR spectrometer. The field setting was 3415 G; the microwave frequency was 9.5 Hz; and modulation amplitudes of 4.0 and 2.0 G were used. Double integration of the electron paramagnetic resonance spectra and comparison with a reference nitroxide at known concentrations indicated that the spin labeled Hb samples contained two labels per Hb molecule to an accuracy of $\pm$ 5%.

Stock solutions of the labeled amino acid ^{19}F-DL tryptophan, and ^{19}F-pyruvate were prepared and aliquots were added to 15% Hb solution to give a final amino acid concentration of 20 mM. For ^{19}F pyruvate, which was used as a control, the final concentration was also 20 mM. For NMR measurements, the samples were first flushed with nitrogen and then transfered to sealed NMR tubes under a nitrogen atmosphere. The ^{19}F-NMR spectra were taken by a Broadband FT-NMR spectrometer JEOL FX-900. The probe temperature was approximately 30°C. Chemical shifts are reported with respect to the chemical shift of free 5 fluorotriacetic acid at pH 7.0 in 10% D_2O. Samples for NMR were prepared in 10 mm NMR tubes. Sample volumes of 5.0 ml were used. All spectra were reported at least three times.

RESULTS

^{19}F NMR Determination of Dissociation Constant and Chemical Shift

The^{19}F NMR spectrum of free 4 DL-fluorotryptophan is shown in Figure 1 and that of 4 DL-fluorotryptophan bound to hemoglobin in Figure 2.

As shown in Figure 2, resonances from three environments are clearly resolved: a broad peak down field and a pair of upfield resonances which together integrate to two times the area of the downfield peak. In each case, the shifts are to a lower field and can be interpreted as evidence that the aromatic ring of 4-fluoro-tryptophan most likely binds in a hydrophobic pocket at the Hb binding site. In the absence of Hb, four resolved resonances with high intensity (Wh 1/2 1.2 Hz) were observed for 4-DL fluorotryptophan, while in the presence of 3 mM Hb at a 4 DL-fluorotryptophan concentration of 20 mM, the spectrum has only three resolved resonances, one of the lower field has Wh 1/2 of 3.2 Hz and the two others have Wh 1/2 of 2.1 Hz.

Incremental addition of ^{19}F DL-tryptophan to a solution of Hb gave a series of chemical-shift values (obs) which were used to derive values for the dissociation constant K of ^{19}F-DL-tryptophan-Hb complex. The final plot of obs against $^{19}F[Trp]_b/^{19}F[Trp]_t$ is shown in Figure 3. The association constant was determined to be 1×10^{-4} M.

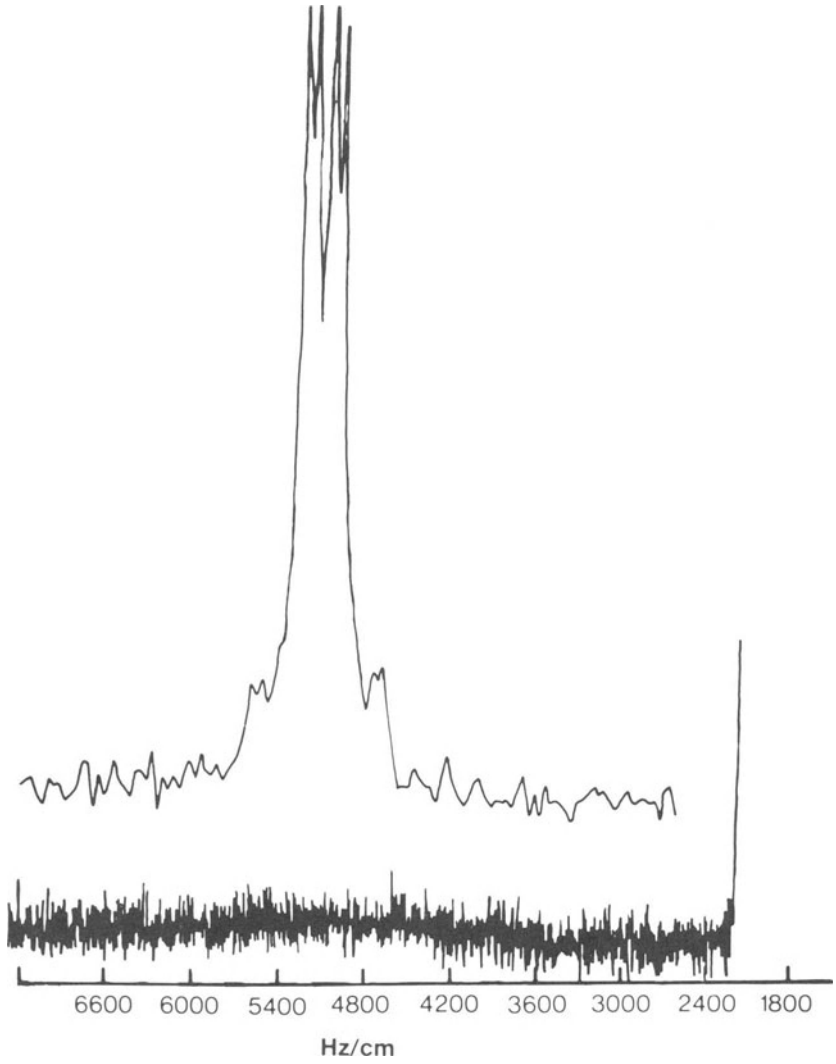


Figure 1. ^{19}F NMR Spectrum of 20 mM 4 DL-Fluorotryptophan. This was determined in 0.05 M potassium phosphate (D_2O), pH 7.0; 350 transients, acquisition time 2.0 sec. The observed splitting of the signal results from the coupling of the fluorine to hydrogens on the indole ring. The chemical shift scale is in parts per million upfield from the signal of trifluoroacetic acid (external capillary). Note that the chemical shift is expanded relative to that of Figure 2.

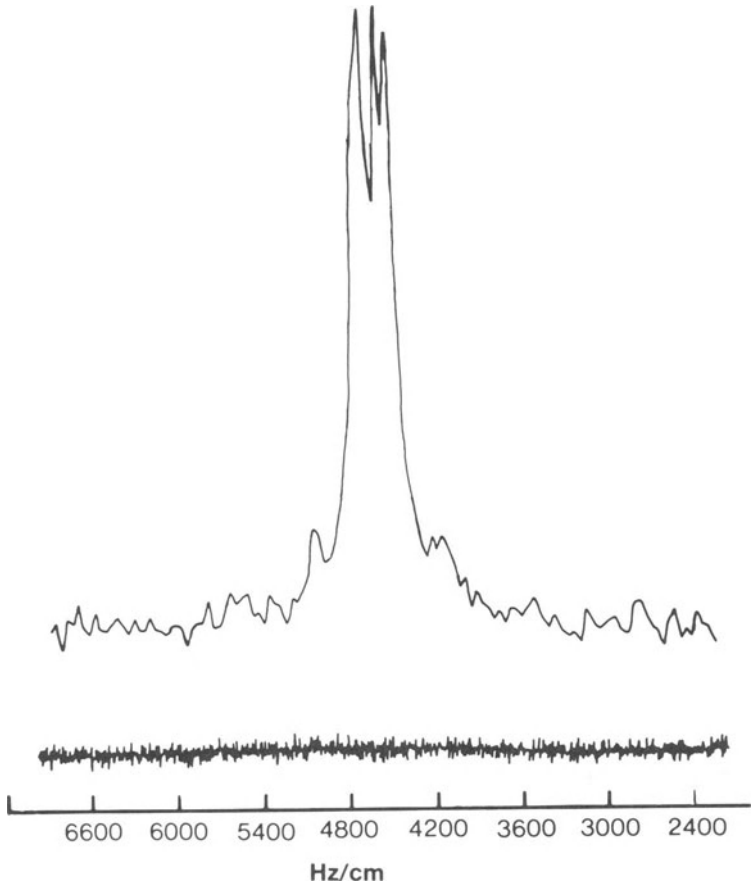


Figure 2. ^{19}F NMR Spectrum of 4 DL-fluorotryptophan-hemoglobin A Complex. It was in a final concentration of 20 mM in 3 mM Hb solution, in 0.05 M potassium phosphate (D_2O), pH 7.0; 3245 transients, and had an acquisition time 0.26 sec.

[^{2}H,^{15}N] MSL Modifications of Either Hb or HbS

The sensitivity and resolution of EPR spectroscopy was substantially improved for biological studies by deuteriation of the frequently used spin label (^{15}N- ^{2}H) MSL which binds covalently to proteins (Zeidan, 1988; Hendrick et al., 1979; Devanx et al., 1981). Significant gains in detectability and resolution were observed with (^{2}H) MSL in both the fast motion limit for freely tumbling spin label (1 x 10^{-10} nsec) and in the slow to very slow correlation time range exhibited by labeled proteins (Seigneuret et al. 1981). The improvement resulted from a reduction in the nonhomogenous line broadening due to the weaker superhyperfine interactions of the unpaired electron with deuterium than with hydrogen. To further advance our capabilities for interpretation of labeled Hb(Hb*) or HbS*, we used the double substituted (^{15}N-^{2}H) MSL to label the cysteine B 93 and to determine the distance between this residue and the tryptophan binding site. It was already known that either N or deuterium substitution increased sensitivity for freely tumbling spin label and that ^{15}N simplified the spectrum by reduction of the number of nuclear mainfolds from three to two lines (Stetter et al., 1976). It was also

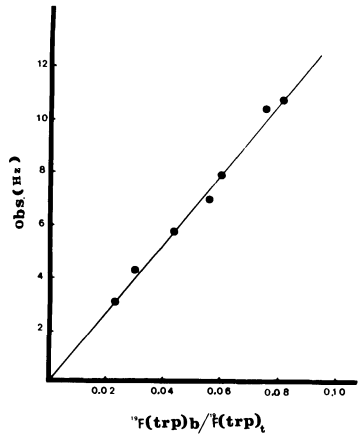
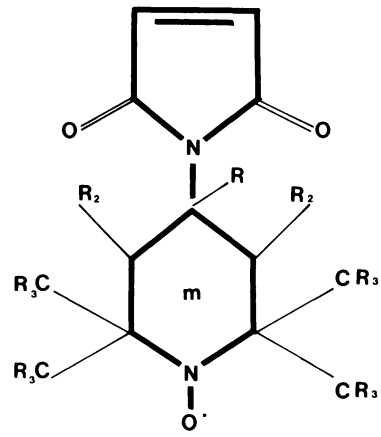


Figure 3. Plot of Observed Chemical Shift Against [Trp]$_b$/[Trp]$_t$ For the ^{19}F Trp-Hb Complex. ^{19}F tryptophan was added incrementally (5-25 mM) to a solution of Hb (3.0 mM) in 0.01 M potassium phosphate buffer, pH 7.4 and the chemical shift (obs) of the signal of tryptophan relative to that of ^{19}F Trp in buffer was observed.

significant in many applications that the signal to noise ratio was increased by a factor of 3 with ^{15}N deuterium labels over a wide correlation time range facilitating studies on smaller amounts of biological materials. In addition, the use of ^{15}N spin labels in double-labeled systems has been suggested as a means to study lipid-protein interactions (Devanx et al., 1981; Stetter et al., 1976). In the present investigation, the use of ^{15}N spin label-^{19}F NMR probe pairs methodology was introduced to examine the intermolecular interactions between probes located in diverse environments. Thus, information on molecular distance between the two sites can be obtained from the spectra (Devanx et al., 1981). In the experiment to be described either Hb or HbS was reacted with (^{15}N-^{2}H) MSL for two hours as described in the experimental section. The spectrum of strongly bound label is shown in Figure 4.

When Hb^{+} at the cysteine B 93 was titrated with 20 mM, ^{19}F-DL-fluorotryptophan, the spectrum shown in Figure 5 was obtained. Spin labeled [^{15}N-^{2}H] MSL attached at the B 93 cysteine residue of hemoglobin induced NMR relaxation of the ^{19}F aromatic moiety of tryptophan with a chemical shift of -48.2 ppm and an overall broadening of the spectrum with a large increase in line width to Wh 1/2 of 12 Hz in the Hb^{+} complex. The large enhancement of T_2 value, verified by complete



(^{15}N,^{2}H) MSL m=15 , R=^{2}H

Figure 4a. Structure of [1-oxyl-2,2,6,6-tetramethyl)-4-piperidenyl] [^{2}H-^{15}N] Maleimide Spin Label.

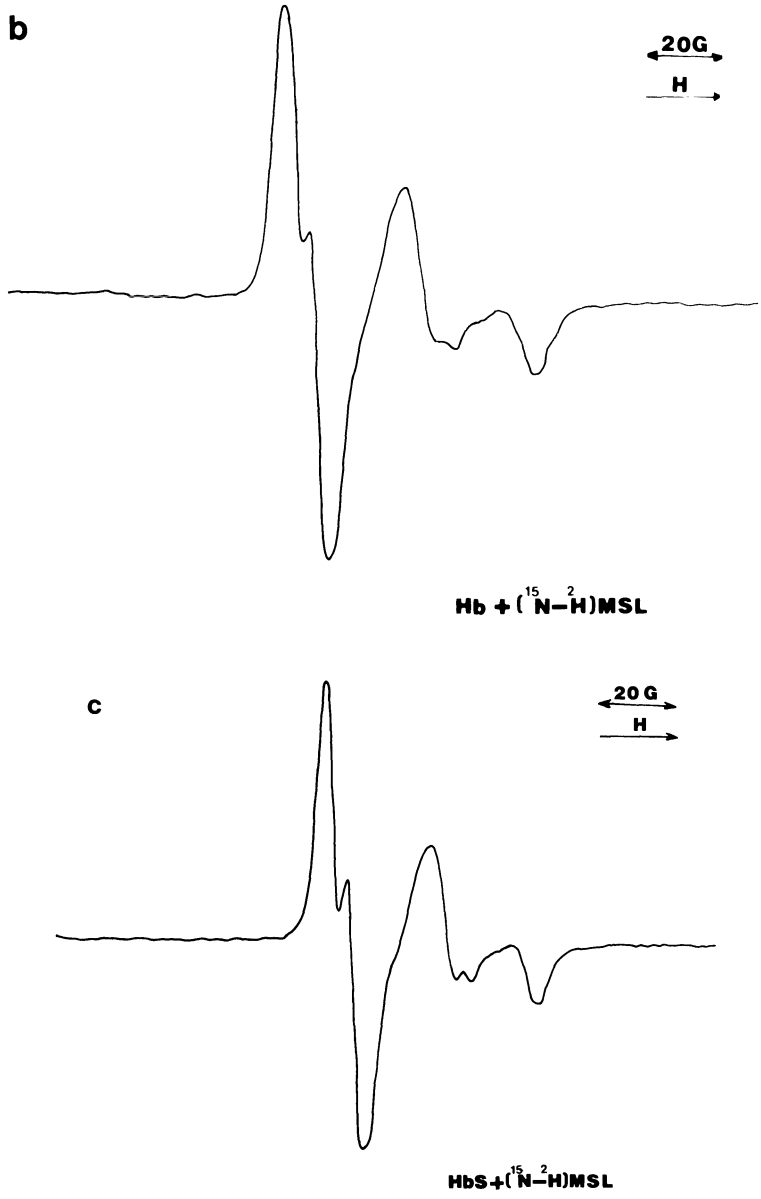


Figure 4b. ESR Spectrum of Hb Reacted With [^{2}H- ^{15}N] Maleimide Spin Label. 3 mM of Hb was reacted with [^{2}H–^{15}N] MSL, total concentration (0.0005 mole) in 1.0 ml of 0.05 M potassium phosphate buffer (pH 7.5). The reaction was allowed to proceed with gentle stirring at room temperature and under anaerobic conditions for 2 hours at 25°C in a nitrogen atmosphere. The hemoglobin solution was dialyzed and the ESR spectrum was recorded. c) ESR spectrum of HbS reacted with [^{2}H–^{15}N] MSL and treated exactly under the same conditions listed in b.

broadening of the spectrum, strongly suggests that the ^{19}F DL-4-fluorotryptophan aromatic binding site is close and is located within 14 of the nitroxide free electron of spin labeled hemoglobin. The ^{19}F spectrum of 4 DL-fluorotryptophan bound to spin labeled sickle

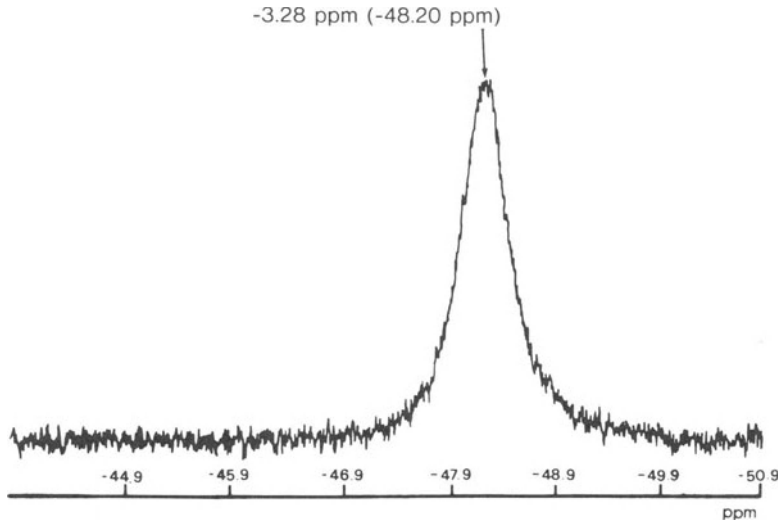


Figure 5. ^{19}F NMR Spectrum of 4 DL-fluorotryptophan Spin Labeled Hemoglobin Complex. Hemoglobin spin labeled with nitroxide spin label MSL to label the two cysteine residues at B 93. The final concentration of hemoglobin was 3 mM in 0.05 M potassium phosphate (D_2O), pH 7.0; 4-DL fluorotryptophan was added to hemoglobin to give a final concentration of 20 mM. The spectrum was recorded at 90 MHz. The chemical shift was referenced to external trifluoroacetic acid, 2119 transients, acquisition time 0.24 sec.

hemoglobin (HbS^*) at the B 93-cysteine group is shown in Figure 6. Again, the spectrum is completely broadened and a large increase in the line width to 28 Hz and chemical shift of -54.0 ppm. The results demonstrate that the 4 DL- fluorotryptophan aromatic moiety binding site is close and within a maximum of 14 apart from the nitroxide free electron of spin-labeled sickle hemoglobin.

When ^{19}F pyruvate was added to either Hb+ or HbS+ at the B 93 sulfhydryl group, no change was observed in the ^{19}F pyruvate spectrum

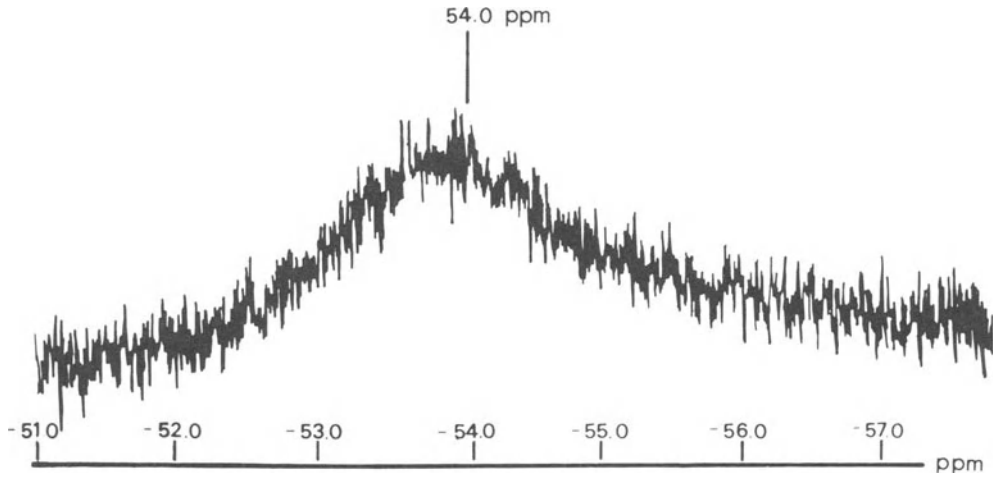


Figure 6. $^{19}_{F}$ NMR Spectrum of 4 DL-fluorotryptophan Spin Labeled Sickle Hemoglobin Complex (HbS^{+}). Hemoglobin samples were spin labeled with nitroxide spin label [^{2}H-^{15}N] MSL to label the two cysteine residues at B 93. The final concentration of hemoglobin was 3 mM sickle hemoglobin in 0.05 M potassium phosphate (D_2O), pH 7.0; 4-DL fluorotryptophan was added to give a final concentration of 20 mM. The spectrum was recorded at 90 MHz. The chemical shift was referenced to external trifluoroacetic acid, 2257 transients, acquisition time 0.29 sec.

(spectrum not shown). These results suggest that probably hydrophobic forces play a dominant role in the interaction of the aromatic moiety of ^{19}F DL tryptophan with either Hb+ or HbS+.

DISCUSSION

The motions of 4 DL-fluorotryptophan bound to hemoglobin and sickle hemoglobin may be investigated by ^{19}F NMR. The spin-lattice relaxation time (T_1), line width (W 1/2), and nuclear overhauser effect (NOE), are related to the molecular motion (Bolton et al., 1981). It would appear from our results that downfield shifts of v = 1.2 to 1.4 ppm for fluorine nuclei attached to the aromatic ring are characteristic of fluoroaromatic amino acids most likely bound at a hydrophobic binding site of Hb. As shown in Figure 2, the line width is especially sensitive to the slower over all motion; thus, the observed line width in Figure 2 indicates that 4 DL-fluorotryptophan is intimately associated with hemoglobin. The

abscence of individual splitting is attributed to a broadening of individual signals. This reflects an immobilization of fluorine nuclei in the 4 DL-fluorotryptophan. Identical results were obtained with HbS. The simple binding behavior observed in our study suggests that the exact location of the aromatic ring in the pocket must depend upon a combination of steric effects, hydrophobic interactions, hydrogen bonding effects and charge transfer interactions.

The theory of paramagnetic effects predicts that the line width of fluorine in the spin-labeled hemoglobin will depend upon r^6 and angular factors while the chemical shift depends uopn r^3 and angular factors. Thus, perturbations to chemical shifts can occur at much longer range than line broadening (Bolton et al., 1981; Bertini and Luchiano et al., 1986). The fact that the spin-labeled hemoglobin and the spin labeled sickle hemoglobin (Figures 4 and 5) give a broad resonance with a relatively short T2 is consistent with a spin label-fluorine interaction within a short distance. A rough calculation can be made after averaging the angular effects to give some approximation of the distance factors of the paramagnetic contribution to line width. This contribution will be at least 10 Hz when r = 8.4 A Bertini and Luchiano, 1986; Sloan et al., 1975), while the absolute value of the paramagnetic chemical shift contributions will be less than 2 ppm only when r is greater than 17 A. The calculated line broadening (W 1/2) of spin-labeled hemoglobin complex (Hb^+) was found to be 12 Hz and 28 Hz with spin labeled sickle hemoglobin (Hb^{++}). Thus, it would appear that the fluorotryptophan binding site is at least 8 A from the immobilized nitroxide label within the hemoglobin. The larger line broadening and greater chemical shift observed with (Hb^+) suggest that the binding sites for both Hb^+ and HbS^+ are similar but not identical. The observed line broadening and chemical shift (Figure 4) suggest a minor conformational change which brought the immobilized nitroxide label closer to the 4 fluorotryptophan. These results confirm our spin label-spin probe investigations (Zeidan, 1988) and further characterize the nature of the contact sites.

When ^{19}F pyruvate was added to either spin labeled hemoglobin (Hb^*) or spin-labeled sickle hemoglobin (HbS^*), no change on ^{19}F pyruvate spectrum was observed. In addition, none of the changes in NMR spectra are observed when either lysozyme or ribonuclease is added as the protein component. These results suggest that hydrophobic forces may play an essential role in the interaction of the aromatic moiety of ^{19}F DL-tryptophan with Hb^+ or HbS^+. Previous investigations of Novak et al. (1978) indicated that the hydrophobic binding site must be near the heme

iron. Therefore, it is likely that Hb and sickle hemoglobin binding site for fluorotrytophan must be in the proximity of the heme iron, most likley within 8-10 of the nitroxide group and also probably include significant hydrophobic moieties.

Previous investigations (Chien, 1979) have indicated that the Mal-6-nitroxide is located within the pocket formed by the F,G,H, and FG segments of the chain. Moffat et al. (1971) suggested that the nitroxide ring displaces the phenol ring of tyrosine 145 and is probably located within the local region formed by residues His B 97, Val B 98, Asp B 99, Leu B 141 and Ala B 142. Baldwin (1980) using the CoHbA crystal coordinated determined the position of the nitroxide radius, and the distance between the nitroxide group and various globin residues. It is believed from these investigations that only the side chains of residues 84-93 in the F-helix of the B- chain are relatively close (8-10 A) to the heme iron. Within this section of the F helix, the side chains of Phe B 85, Leu B 88, and Leu B 91 all project out toward the surface of the Hb molecule and form a localized hydrophobic pocket close to the heme ring. X-ray crystallographic studies by Wishner et al. (1975) have reported the involvement of these residues in the aggregation of deoxy HbS. Previous investigations (Wishner et al., 1976; Hagen et al., 1970) have shown that the B mutation contact site (Glu-Val) contains residues Phe B 85 and Leu B 88 which are in Van der waals contact with the Val B 6 side chain. Therefore, it is likley that the inhibition exhibited by tryptophan and other aromatic compounds essentially results from competitive binding at the B 85, B 88 region. Furthermore, we suggest that the hydrogen binding and van der waals contact between the aromatic fluorine and Phe B 85, Leu B 88 at the bottom of the aromatic binding pocket may be also an important interaction responsible for orienting the aromatic moiety laterally within its binding site. Further investigations are now in progress to prove this hypothesis and to determine the exact location of the aromatic ring in the pocket, and the geometry of the aromatic binding site.

SUMMARY

The binding of ^{19}F DL-4-tryptophan to hemoglobin has been studied by ^{19}F nuclear magnetic resonance spectroscopy. The results suggest that ^{19}F-tryptophan exhibits hydrophobic binding to hemoglobin. Site-directed paramagnetic nitroxide radicals (spin labels) have been used to perturb the ^{19}F - NMR spectra of bound tryptophan.

Hemoglobin and sickle hemoglobin spin-labeled at the B 93 cysteine residue broaden the ^{19}F NMR spectrum of tryptophan bound at the hydrophobic site. The broadening of the spectrum suggests that the binding site is located within 8-10 A of the nitroxide free radical of spin-labeled hemoglobin. This binding site is close to the heme iron and mainly involves a group of hydrophobic residues on the surface of the chain. The resonance data are in excellent agreement with our recent investigations and with the model built according to the X-ray crystallographic coordinates of Wishner et al. (1975).

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The Active Site of Monoamine Oxidase "The Key Enzyme of Biogenic Amines Degradation" as Seen by Magnetic Relaxation Spectroscopy

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INTRODUCTION

In the central nervous system, monoamine oxidase (MAO; EC 1.4.3.4) is postulated to be important in controlling the levels of certain neurohormones and other pharmacologically important amines (Sandler et al., 1973; Von Korff, 1979). The potential importance of the enzyme in neuropharmacology has prompted us to explore the nature of the active site by spin labeling techniques. Monoamine oxidase has been characterized by amine substrate specificities and response to inhibitors as having two independent catalytic sites (Tipton et al., 1981; Jani, 1977) or as two distinct enzyme forms (A,B) embedded in the same mitochondrial membrane (White and Tansk, 1979; White and Stine, 1982). Other authors postulated that MAO-A and MAO-B may be the same enzyme in different lipid environments (Huang, 1980; White and Stine, 1982; Fowler and Ross, 1984). The MAO-B which preferentially deaminates benzylamine is sensitive to deprenyl inhbition. Tyramine and tryptamine are substrates for both MAO-A and MAO-B enzymes (Tipton et al., 1976; Oreland and Eksted, 1976). Many of the complex problems surrounding the mechanism of action, regulation and substrate specificity of mitochondrial MAO have resisted definitive resolution when explained by traditional enzymological approaches. We have been exploring the utility of spin labels in studying these problems. Of particular interest is the controversial question of the function and structural basis of the enzyme multiplicity of substrate specificities (White et al., 1979; Oreland and Eksted, 1976). As a first step toward a three dimensional model of bovine liver monoamine oxidsae B, we have embarked on a specific study of the environment of the essential cysteine residues using conventional ESR spin labeling techniques. The details of this aspect of MAO research have been elaborated elsewhere (Zeidan et al., 1979). As a continuation

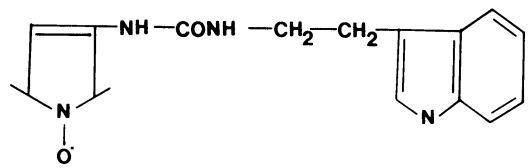
of our efforts to verify the nature of the active site we have embarked upon a specific study to probe the active site by using a suitably chosen labeled substrate, tryptamine. The ESR spectra of the label in the presence of an enzyme provides a convenient method to characterize structural features of the active sites in vitro and to provide a clue as to the nature of MAO's active site.

MATERIALS AND METHODS

Materials

Highly purified bovine liver MAO was obtained following the procedures of Watanabe et al. (1980). The specific activity of the enzyme was 10,000 U/mg. The purity of the enzyme was checked by SDS polyacrylamide gel electrophoresis. Only a single band was observed at a molecualr weight of 52,000 g mol^{-1}. Spin labeled tryptamine [N-(1-oxyl)-2,2,5,5-tetramethyl pyrroline-3-yl)-N-(3-B-ethyl) indolylcarbamide] (TrySL) (Figure 1) was purchased from Medimpex (Budapest, Hungary). The other chemicals were reagent grade and purchased from standard chemical companies.

The enzyme was assayed by the spectrophotometric procedure of using benzylamine as a substrate (Tabor et al., 1954). Electron paramagnetic resonance (EPR) measurements were recorded at room temperature by a Varian EPR spectrometer. The field setting was 3415G. The microwave frequency was 9.5 hZ, and modulation amplitudes of 4.0 and 2.0 G were used. The MAO-TrySL was prepared by the addition of TrySL dissolved in 0.01 M phosphate buffer, pH 7.5 directly to MAO in the same buffer. Titrations of MAO with TrySL were performed by successive additions of 0.005 ml increments of 5 x 10^{-3}M TrySL. The MAO concentration was 3.8 x 10^{-5}M. The concentration of TrySL was determined by computer-assisted double integration of spectra and comparison with a standard solution of known concentration. In the case of a low concentration of MAO bound



SL TRYPTAMINE

Figure 1. Structure of Spin Labeled Tryptamine Methods.

label, quantitative measurements were performed from the decrease of the amplitude of the free label following addition of the substrate analogue. The concentration of paramagnetic molecules was used in all calculations of dissociation constants. All solutions contained NaCN to retard extraneous nitroxide reduction.

The longitudinal relaxation rate $1/T_1$ (T_1 is spin lattice relaxation time) of water proteins in solutions containing analog and/or MAO was measured with a precison of $\pm$ 1% by a saturation recovery method at 250 MHZ (Freeman and Hill, 1971). The 90° pulse width was determined to be 100 us. Relaxation rates were calculated from the relaxation curve using non-linear least square methods. Each relaxation curve contained at least ten intensity measurements at appropriate pulse intervals. Statistical uncertainities in the individual rates were generally about 10% of the calculated values. All the data in all tables and figures were the average of 4 replicates.

RESULTS AND DISCUSSION

ESR Spectrum of Spin Labeled Tryptamine With MAO

The specificity of binding between spin-labeled tryptamine and MAO was carefully investigated. The addition of TrySL to a highly purified enzyme preparation caused the amplitudes of all three lines of the paramagnetic resonance spectrum to decrease. The observed changes in the ESR spectrum were indicative of binding. Comparison of the amplitude of each line with that of the corresponding line in the standard yields unequal estimates for the percent radical bound to the enzyme. The largest change in amplitude relative to the standard was observed for the high field line with intermediate values for the low field line and lowest values for the middle field line. Such broadening can be attributed to decreased motional freedom of the bound state for a spin label interaction with a protein (Figure 2). The spectrum indicated relatively fast and nearly isotropic motion of the spin label suggesting that the bound tryptamine analogue is in a "partial immobile state" and that the catalytic sites are close to the surface of the enzyme (Zeidan et al., 1980). Calculation of the correlation time which reflects the environment of the active site of the enzyme is straight forward since there is only one type of motional freedom. It can be determined using equation 1 (Likhtenshtein, 1976; Zeidan et al., 1985) where H is the peak to peak width of the central field line (in G), H_0 and H_{-1} are peak to peak heights of the central field and high field line, respectively. The calculated rotational correlation time was found to be 6.2×10^{-9} sec. The corresponding $_c$ (correlation time) for TrySL in buffer would be of the order 10^{-10} sec.

Equation 1

$$\frac{1}{Tc} = \frac{3.6 \times 10^{-9}}{(\frac{ho}{h-1}) \Delta H_o}$$

Selective binding of spin-labeled tryptamine to MAO was examined by using other proteins such as bovine serum albumin, ribonuclease, and lactate dehydrogenase at concentrations comparable to that in the highly purified MAO preparations. The ESR signal indicated no binding of spin labeled tryptamine to any of these proteins (spectrum not shown). There was no detectable interference with the assay from the Triton X-100 which was present at a very low concentration in the enzyme preparation during the determination. That MAO bound spin labeled tryptamine reversibly was clearly indicated by the lack of a residual observable ESR signal after a mixture of MAO-spin label complex was dialyzed against 0.01 M potassium phosphate, pH 7.5.

Equation 2

$$\varepsilon = \frac{(\underline{Try.SL})_f}{(Trp.SL_t} + \frac{(\underline{Try.SL})_b}{(Try.SL)_t}$$

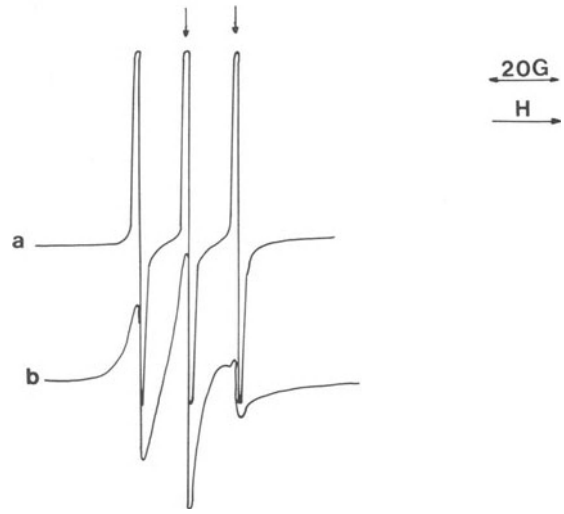


Figure 2. Paramagnetic Resonance Spectra of TrySL in a) 0.01 M Potassium Phosphate Buffer, pH 7.5 (1 x 10^{-5}M. b) the Presence of MAO. The enzyme (2.0 mg in 1.0 ml of 0.01 M potassium phosphate, pH 7.5) was reacted with 1.0 x 1^{-} (final concentration) of TrySL under anaerobic conditions at pH 7.5 and room temperature (see Materials and Methods section.

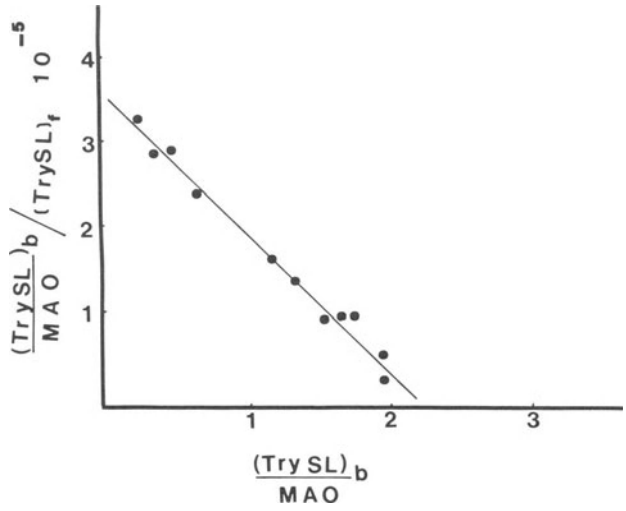


Figure 3. Schatchard Plot of the Paramagnetic Resonance Data for TrySL Binding to MAO. The line intersecting the abscisa at (TrySL)b /Et = 2.2 was determined by a least square fit of the data.

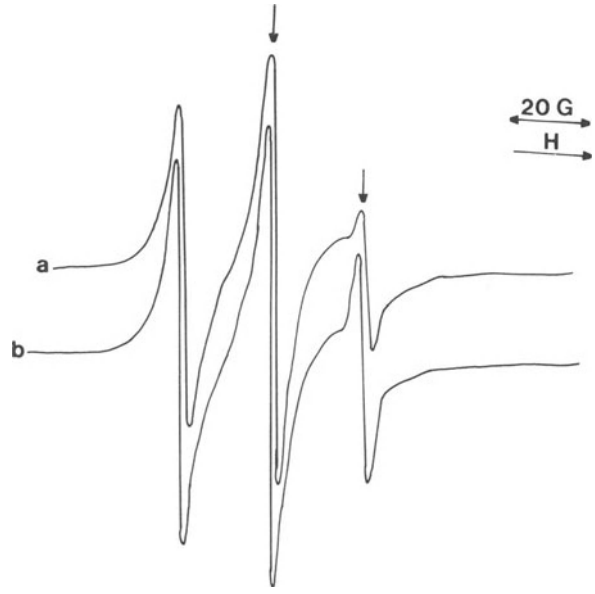


Figure 4. ESR Spectrum of the Enzyme. a) Same as 2c, b) MAO reacted with 2.5 x 10^{-3}M benzylamine then reacted with TrySL. The same gain setting as in a.

Where the subscripts f, b, and t refer to the concentrations of free, bound, and total radical tryptamin, respectively. By definition ε^* = 1. ε_b may be calculated from equation 2 using the value for the fraction $(TrySL)_b$ bound obtained from paramagnetic resonance measurements. The data presented in Table 1 show that ε_b =3.2, that is the bound radical is 3.2 times more effective in relaxing water protons than the unbound radical. The observed is about one half of that for a strongly immobilized covalently-bound nitroxide (Taylor et al., 1989). Although the enhancement is a function of the access of water to the nitroxide, as the extent of immobilization of the radical and its degree of hydration, this relatively low value is consistent with the partially immobilized state of the bound TrypSL, indicated by the paramagnetic resonance spectrum.

Displacement of (TrypSL) From Monoamine Oxidase by Substrates and Inhibitors

When increasing concentrations of tryptamine were added to a solution of (TrypSL) and MAO, the bound (TrySL) was progressively displaced from the enzyme (Figure 3). These results are illustrated in Table 2 for tryptamine. The data show a progressive decrease to a limiting value of K_D as the tryptamine concentration is raised. This change may be attributed to a variation in affinity with site occupancy. Tryptamine appears to be competing for the same site on the enyzme, and the dissociation constant for tryptamine (38 m) was approximately the same as that for TrySL.

Preincubation of the enzyme with benzylamine increased the amplitude of the free label (Figure 4). As benzylamine incubation should compete with the probe for the active site, one would expect a larger effect on $(TrypSL)_b$. Thus, the possibility of some accessibility to the MAO active site by other amines and the multiplicity of the catalytic sites of the enzyme are highly suggested.

Polarity of the Active Site

Another parameter obtained from the ESR spectrum on the nitroxide spin label, useful in characterizing their enzyme bound environment, is the distance between the three spectral lines (hyperfine splitting). This parameter characterizes the unpaired electron density of the nitroxide nitrogen and is highly sensitive to the polarity of the probe environment, decreasing for more hydrophobic surroundings. For spin labeled tryptamine bound to MAO, the spectrum has a line shape indicating that there is some restriction of motional freedom (i.e., the spectrum is

Table 1. Effect of TrySL and Its Complex With MAO on the Proton Relaxation of Water.

Added to 0.01 M Phosphate Buffer (conc, mM)	$1/T_1$ or (Sec 1)	$1/T_{1p}$ (Sec 1)	a	$[TrySL]_b$ / [TrySl]t	b
None	0.403	-	-	-	-
MAO [d] (0.211 mM)	0.412	-	-	-	-
TrySL (0.589 mM)	0.548	0.143	-	-	-
TrySL (1.02 mM)	0.692	0.285	-	-	-
TrySL (2.04 mM)	1.015	0.602	1.02	-	1.02
TrysL (0.589 mM)+	0.729	0.302	1.79	0.628	3.4
MAO (0.211 mM)	-	-	-	-	3.2-3.4

[a]Calculated from the definition of ε *.

[b]Determined by EPR.

[c]Calculated from a and b using equation 2.

[d]MAO site concentration assuming 2.0 sites per enzyme monomer.

[e]Average of 12 determinations.

Table 2. Displacement of TrySL from MAO by Tryptamine and Calculation of the TrySL-MAO Dissociation Constant[a].

Tryptamine mM	Free TrySL (%)[b]	Calculated K_D, Try (10^{-6}M)
0.142		
0.252	22.4	
0.415	29.6	31.4
0.621	37.6	28.9
0.809	45.2	25.8
1.120	58.9	21.9
1.680	69.4	19.6
1.980	76.4	17.9
2.510	82.4	15.1

[a]MAO site concentration and $[TrySl]_t$ were 4.2×10^{-5}M and 5.4×10^{-5}M, respectively, in 0.01 M potassium phosphate, pH 7.5.

[b]Determined by electron paramagnetic resonance.

not completely isotropically averaged). In such a case, the peak separation can not be taken as the isotropic coupling constant. However, by increasing the tryptamine and benzylamine concentrations, this slight restriction is demolished, and the value for the hyperfine splitting is 16 ±1 gauss, while the measure for free labeled tryptamine in buffer is 15 ±1 gauss. Thus, the average environment of the label in the presence of the enzyme does not appear to be appreciably hydrophobic. Rather, it appears that the environment of the active site is most likely hydrophilic in nature.

SUMMARY

The spin label substrate, tryptamine, was used as a structural probe of the active site of bovine liver monoamine oxidase B. When the reaction was monitored by electron spin resonance (ESR), line broadening effects indicative of binding with an apparent relation to substrate specificity of the highly purified enzyme were observed. The spectrum indicated that the bound tryptamine was "partially immobilized" with a dissociation constant of 38 m and 2.2 mole bound per enzyme dimer. The bound radical enhanced the longitudinal relaxation rate of water protons by a factor of 3.2. The environment surrounding the catalytic site and the mobility of the label are both characterized and discussed.

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Spectral Studies on Monoamine Oxidase Modified with N-(1-Pyrene) Malemide Fluorescent Probe

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INTRODUCTION

Monoamine oxidase is involved in the control of the biogenic amines degradation in the brain and central nervous system (Singer et al., 1979; Usdin, 1976). This involvement has stimulated considerable interest in the physiocochemical and enzymatic properties of the enzyme. The enzyme contains four sulfhydryl groups per subunit of enzyme only one of which is thought to be essential (Oi et al., 1971). Physiochemical studies have indicated that the enzyme is made up of two identical subunits (Igaue et al., 1969). From the effect of pH on the kinetic properties of the enyzme, it was concluded that a cysteine residue in the enzyme is catalytically important in the breakdown of amines. Gomes et al. (1969) have shown that 5,5'dithiobis 2-nitrobenzoic acid (DTNB) reacts with several cysteine residues and causes inactivation of the enzyme, however, the study concluded that only one sulfhydryl residue was essential per subunit of the enzyme. It was suggested that this sulfhydryl residue may be a component of the active site. As a first step toward a three-dimensional model of bovine liver monoamine oxidase-B, Zeidan et al. (1980) embarked upon a specific study of the environment of the sulfhydryl residue using conventional ESR spin-labeling techniques. The details of this aspect of MAO research have been elaborated elsewhere (Zeidan et al., 1980).

As a continuation of the efforts to verify the local environment of the essential sulfhydryl group,, this laboratory has begun a specific study to probe the nature of the active site by using an extrinsic pyrene probe. In the work described here, we have used an extrinsic covalently attached label, N-(1-pyrene) maleimide, a specific sulfhydryl reagent that becomes fluorescent only after reacting with a sulfhydryl group (Wu et al., 1976). This fluorophore offers the advantages of a high quantum

yield for easily detectable signal, clear excitation and emission spectra, and a sufficiently long fluorescent life time. This probe has been used in this study to monitor any conformational changes which took place in the local environment of the essential sulfhydryl groups during the pH change, urea denaturation and increasing ionic strength.

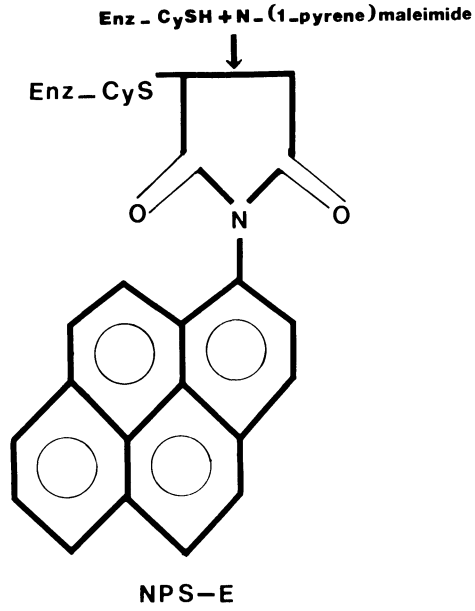
MATERIALS AND METHODS

Materials

Highly purified enzyme was isolated following the procedure of Watanabe et al. (1980). The specific activity of the enzyme was 10,000 μ/mg. The purity of the enzyme was checked by SDS polyacrylamide gel electrophoresis. Only a single band appeared at a molecular weight of 52,000 g/mole. N-(1-pyrene) malemide was purchased from Molecular Probes. The other common reagents used were of reagent grade and were purchased from standard companies.

Methods

Modification of Bovine Liver. Modified enzyme derivative (in which the nonessential sulfhydryl groups were blocked using iodoacetate) was synthesized following the procedure of Zeidan et al. (1980).



Fluorescent Labeling of the essential sulfhydryl group on MAO

Figure 1. Reaction Scheme of NPM With a Cysteine Residue.

Fluorescent Labeling of the Essential Sulfhydryl Groups

The essential sulfhydryl group of the enzyme was labeled by reacting the modified enzyme derivative with NPM to form the corresponding succinimido-MAO adduct (Figure 1). Modification of the enzyme with NPM was carried out by applying 5 x 10^{-4} moles NPM in 1.0 ml of dimethyl sulfoxide to a Whatman filter paper in a test tube which was then dried by a stream of nitrogen. Then, 9.5 x 10^{-6} moles of modified enzyme dissolved in 1.0 ml of 0.05 M potassium phosphate buffer, pH 7.5 was added to the test tube. The reaction was allowed to proceed with gentle stirring at room temperature and under anaerobic conditions. It was then exhaustively dialyzed against 0.05 M potassium phosphate buffer, pH 7.5.

Amino acid analyses were performed on 24 hour acid hydrolyzates (110°C), after the samples were passed through a 1.5 x 10 cm column of Sephadex G-25, on a Beckman Model 121 MB automatic amino acid analyzer as described by Tarr (1985).

Spectral Measurements

Fluorescence excitation and emission spectra were recorded with a Perkin-Elmer MPF-4 fluorescence spectrophotometer using a 1.0 cm path cuvette. Excitation was at 330 nm for NPM fluorescence. Emission spectra were recorded at room temperature unless otherwise stated. The experiments are described in the legends.

RESULTS

Modification of MAO

The enzyme was modified by reaction with iodoacetic acid as described in the experimental section and by Zeidan et al. (1980). Three nonessential cysteine residues per subunit of enzyme reacted within 6 hours at pH 7.4 and 25°, with little loss in enzyme activity (Figure 2). As the remaining essential cysteine residues reacted, activity dropped progressively and after 24 hours, only 5% activity remained. From this investigation, we concluded that iodoacetate can be used to block the nonessential sulfhydryl groups.

NPM-MAO Interaction

NPM did not fluoresce or form a fluorescent product in solution alone. Under the conditions described under experimental methods, the MAO derivative was first synthesized in which the six nonessential sulfhydryl groups per dimer were blocked. This derivative was then reacted with fluorescence label NPM. This then attached the label on the essential sulfhydryl groups. After dialysis to remove excess reagent, the fluorescence spectrum of NPM-MAO adduct was recorded (Figure 3). The fluorescence spectrum exhibits two major vibronic bands at about 375 and

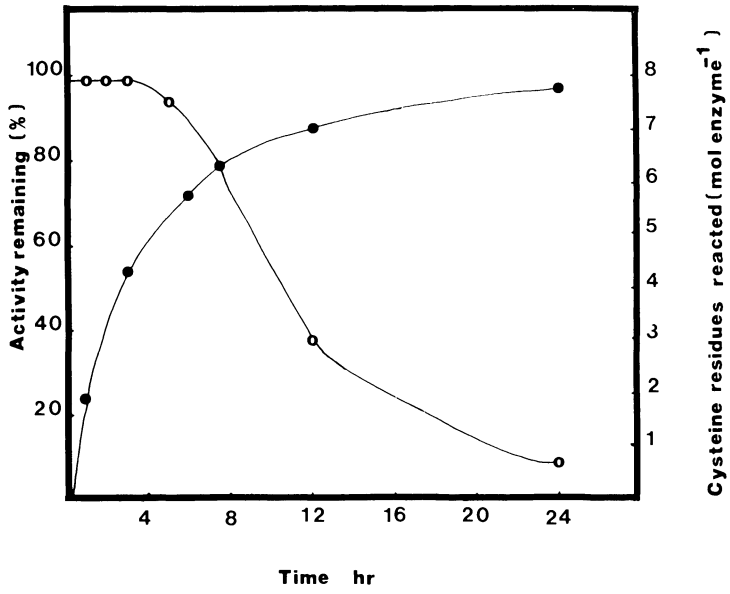


Figure 2. Effect of Iodoacetate on the Activity of Monoamine Oxidase. The enzyme was modified and assayed for activity as described in the experimental section. (This figure was taken from Zeidan et al. (1980).

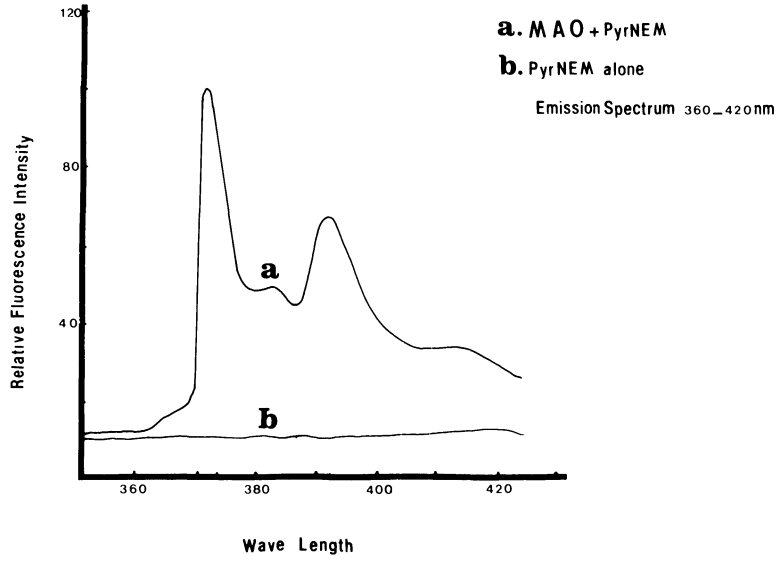


Figure 3. Fluorescence Emission Spectrum of NPM in the Absence and Presence of MAO. Fluorescence was recorded in 1.0 ml of 0.01 M KPB, pH 7.5. Both the excitation and emission slits were set at 7 mm. The sample sensitivity was 30. The protein concentration was 0.087 mg/ml. The NPM labeled enzyme was dialyzed against 0.01 M KPB, pH 7.5, for 24 hours before recording the spectra.

395 nm and a shoulder at 415-420 nm. It closely resembles the spectra of several NPM-labeled proteins reported by Weltman et al. (1973) but is shifted slightly to the blue with respect to them.

When MAO derivative was treated with nonfluorescent sulfhydryl reagent N-ethylmaleimide before NPM, their fluorescence intensity was diminshed by about 95%, indicating that the two reagents compete for the same sites. When MAO was treated with NPM, the fluorescence intensity was enhanced suggesting that the label had reacted with some of the nonessential sulfhydryl groups.

The pyrenemaleimide adduct with MAO exhibited two distinctive fluorescent components at about 375 and 395 nm. As a working hypothesis, we would like to report that pyrene maleimide is quite sensitive to one or more specific factors which define the microenvironment about the protein bound chromophore. What these factors may be is not known at present. Since the pyrenemaleimide molecule is not symmetrical with respect to the position of thiol attack, the two spectroscopic components may be a reflection of the two ways in which the pyrene may rest on the protein surface, that is with either the bulk of the pyrene ring buried and the succinoamide moiety exposed to the water environment or vice versa. The extent to which the pyrene is buried or exposed would be due to the specific environment of each sulfhydryl group, which would differ for each protein. As in the case of pyrenemaleimide label, Zeidan et al. (1980) using maleimide spin label approach have studied the origin of the motionally heterogeneous components in the electron paramagnetic resonance spectrum. Zeidan et al. (1980) concluded that this heterogeneity may arise from an equilibrium between two conformational states that can be arrived at by the spin label being on a single amino acid residue, with the two different conformations leading to different motional freedom. Furthermore, they concluded that the essential sulfhydryl group is buried into a shallow pocket in the protein. The use of pyrenemaleimide fluorescent label seems to support the previous EPR findings and further characterizes the local environment of the essential sulfhydryl groups.

Influence of Urea on the Pyrenemaleimide Spectrum

As previously stated NPM by itself is not fluorescent, but becomes so after reacting covalently with the sulfhydryl group of a large or small molecule (Weltman et al., 1973). Emission spectra of both kinds of NPM are shown in Figures 3 and 4. The large molecule being the MAO derivative (Figure 3) and the small, 2-mercaptoethanol (Figure 4). Differences appeared when 6 M urea was present in the medium. The emission of NPM-labeled MAO derivative was markedly quenched (Figure 5).

The different emission bands were quenched unequally. The major band at 375-6 nm was reduced to less than half its former intensity; the 395-7 nm band and the shoulder at about 385 nm were also quenched, but less. In contrast, the emission of NPM adduct of meracaptoethanol was not quenched by 6 M urea (Figure 4). The quenching by urea of MAO-NPM fluorescence cannot, therefore, be due only to solvent-fluorophore interaction, but must be at least partly due to urea-induced conformational changes in the protein. The conformational change induced by urea was irreversible at 6 M urea. Unfolding and exposure to urea cause massive structural changes in the MAO and irreversibly abolish its activity at 6 M urea (Figure 6). Thus, loss of activity by denaturation paralleled loss of pyrenemaleimide fluorescent signal.

Influence of Ionic Strength on the Pyrenemaleimide-MAO

One of our main reasons for undertaking this work was to attempt to detect smaller conformational changes, closer to those which may take place under physiological conditions. For this purpose, we examined the effect of increasing the concentration of potassium chloride on both

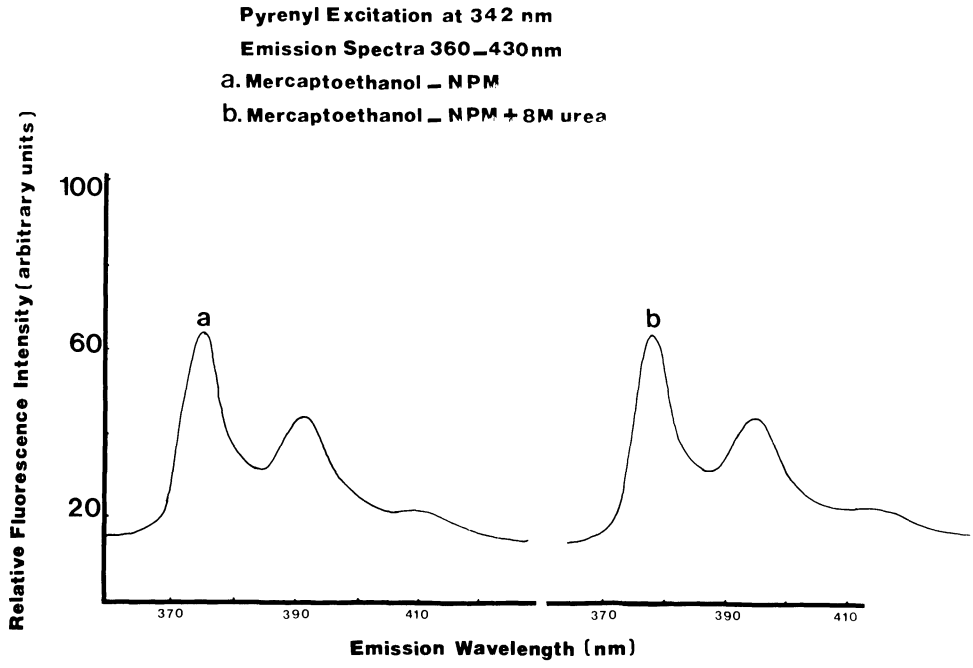


Figure 4. Effect of 8 M Urea on the Fluorescence Emission Spectrum of Mercaptoethanol. Excitation and emission slits were set at 7 mm. The sample sensitivity was set at 30.

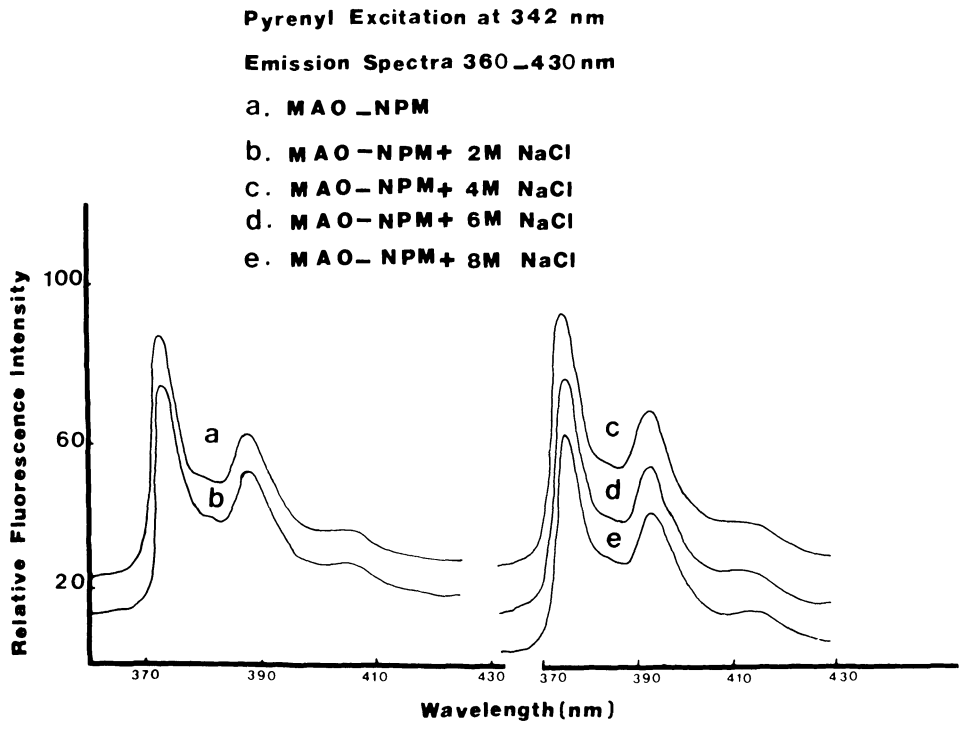


Figure 5. Effect of Urea Concentration on MAO-NPM Fluorescence. The enzyme was modified as described in experimental methods section. The NPM labeled enzyme was dialyzed against 0.01 M kPB, pH 7.5, for 24 hours before recording the spectra.

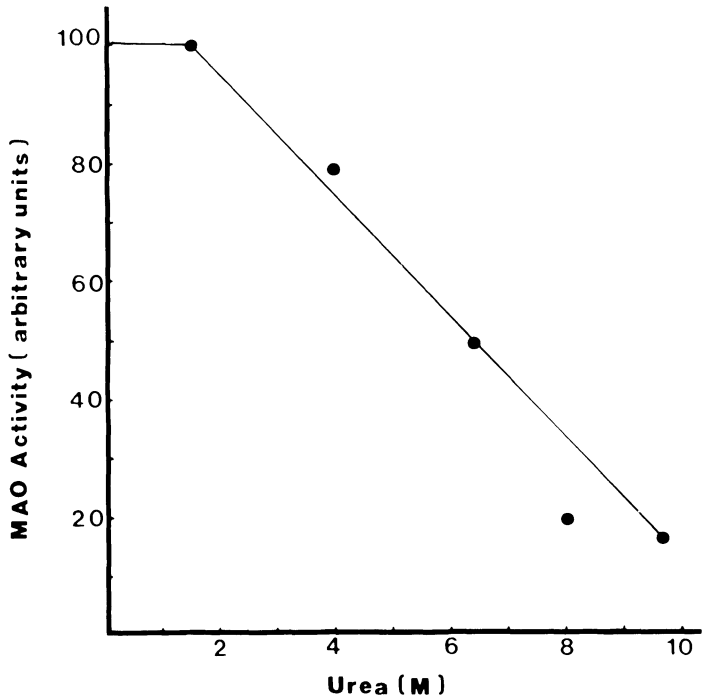


Figure 6. Effect of Urea concentration on the Activity of MAO. The enzyme was treated first with urea then assayed for activity as described in the experimental section.

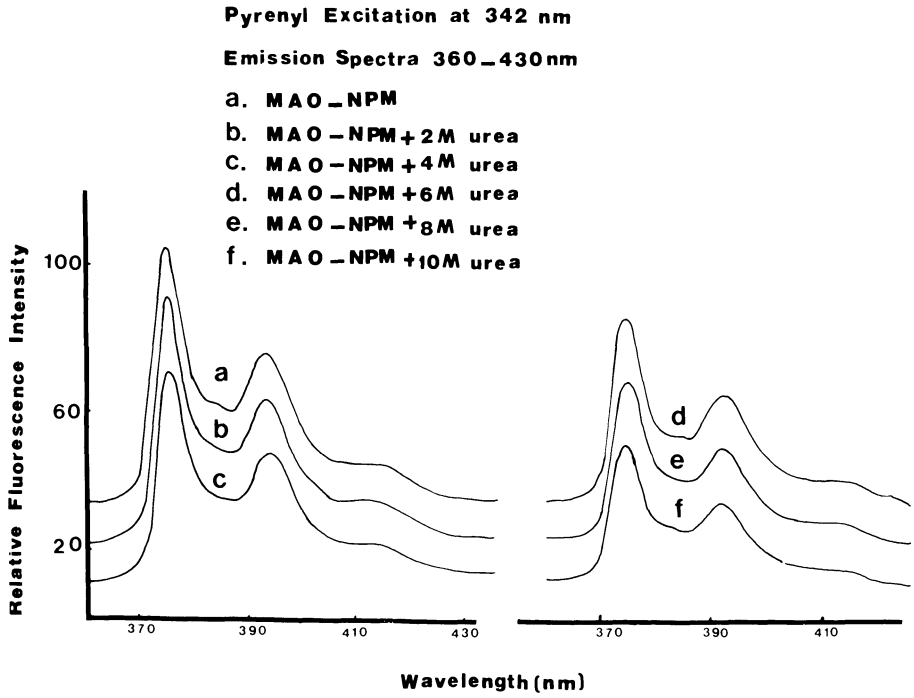


Figure 7. Effect of Varying Ionic Strength on MAO-NPM Fluorescence. The enzyme was treated with sodium chloride and modified with NPM as described in the experimental section. The NPM labeled enzyme was dialyzed against 0.01 M KPB, pH 7.5, for 24 hours before recording the spectra. Both excitation and emission spectra were set at 7 mm.

pyrenemaleimide-MAO fluorescence spectrum and MAO activity. Increasing ionic strength appeared to have a very small effect on both the fluorescence spectrum of the adduct (Figure 7) and activity of the enzyme. Only a 5% increase in activity was observed at 0.8 M potassium chloride.

Influence of pH on the Pyrenemaleimide-MAO Spectrum

The effect of varying the pH solutions of pyrenemaleimide-MAO derivative are shown in Figure 8. As seen in the spectrum, there is a gradual change in the spectrum from pH 7 to 11. Activity studies have demonstrated that the enzyme maintains 75% of its activity within the range of 7-9 and loses about 90% of its activity at pH 11.5. Thus, loss of activity with pH change parallels loss of the pyrenemaleimide-MAO derivative signal. As has been previously reported (Oi et al., 1971), an essential sulfhydryl residue with a pKa of 10 is involved in breakdown of the amine. The fluorescence data reported here with the activity studies

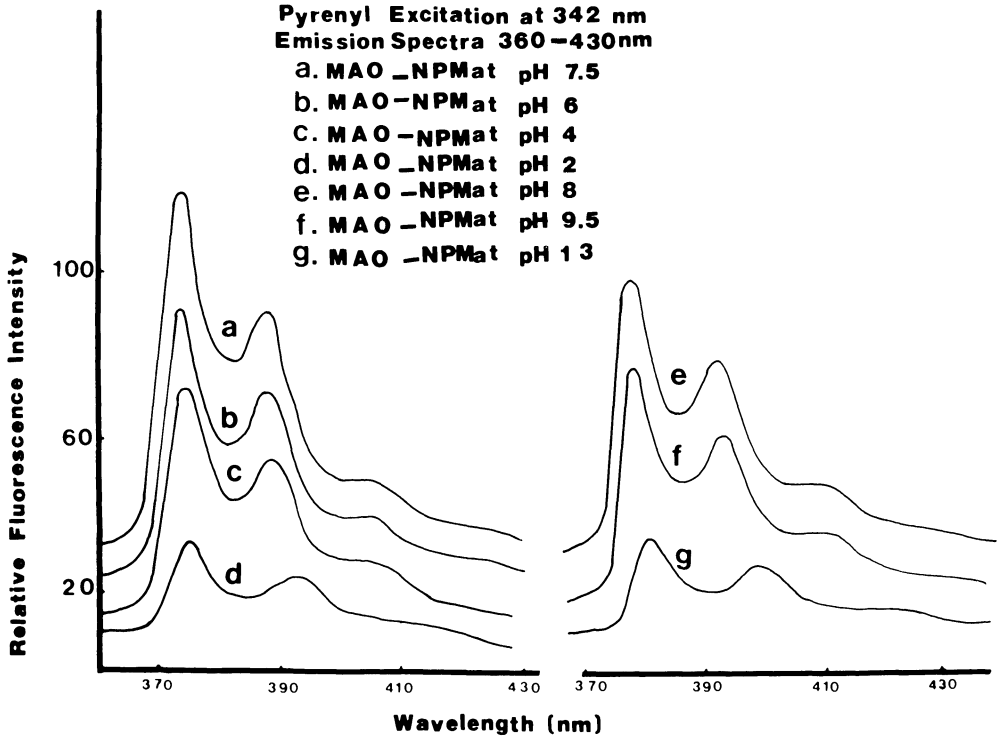


Figure 8. Effect of pH on MAO-NPM Fluorescence. The enzyme was modified and labeled at the indicated pH as described in the experimental section. The NPM labeled enzyme was dialyzed against 0.01 M KPB, pH 7.5 for 24 hours before recording the spectra. Both excitation and emission spectra were recorded at 7 mm.

seems to support the suggestive evidence of the importance of the sulfhydryl groups and charactarize the local environment where this residue is located.

SUMMARY

A pyrenemaleimide attached to the essential sulfhydryl groups of monoamine oxidase B has been used to probe the structure of the active site of the enzyme. The label is nonfluorescent in aqueous solution but forms a strongly fluorescent adduct with the essential sulfhydryl group of the enzyme. The extrinsic probe was found to be sensitive to a limited and fully reversible transition that took place when the enzyme was incubated under conditions that changed its activity in vitro. This suggests that N-(1-pyrene) maleimide label may be used to probe both the environment of the essential sulfhydryl group and the local conformational changes that occur under conditions consistent with activity.

Therefore, we conclude that the extrinsic probe NPM is able to detect conformational changes at the active site. Furthermore, it can be used to determine the microenvironment of the proteins that carry the bound NPM molecules.

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The Infrared Spectra of Buried Acetate and Rayon Fibers

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INTRODUCTION

Textile fibers are frequently found as trace evidence in criminal investigations. The term "trace evidence" refers to any microscopic or submicroscopic evidence that may be exchanged between the scene of a crime and its perpetrator (DeForest et al., 1983). In addition to textile fibers, trace evidence may include glass, soil, human and animal hair, paint chips and smears and bits of plastic. Such evidence may be very important in identifying the perpetrator of a crime or in reconstructing the crime.

Textile fibers can be divided into two categories: natural and man-made. Natural fibers include cellulosic fibers (plant-derived material such as cotton and flax), proteinaceous fibers (animal-derived material such as wool and silk) and inorganic fibers (such as asbestos). Man-made fibers consist of regenerated fibers (e.g. rayon and cellulose acetate, which are produced from naturally occurring material) and synthetic fibers (e.g. polyesters, acrylics and polyamides, all of which are produced ultimately from petrochemicals). Natural fibers can be identified by their microscopic appearance. They may have surface scales and a complex internal structure. Man-made fibers, on the other hand, are much harder to identify because they generally have no distinctive internal morphology. Because microscopy is non-destructive, it remains the most important method for identifying man-made fibers. The composition of a fiber may be determined from its indices of refraction, birefringence or dispersion staining colors, all of which may be obtained with appropriately equipped light microscopes. Man-made fibers may also be identified by their solubilities in various acids and bases and in various organic solvents (Federal Bureau

of Investigation, 1978). Instrumental methods of analysis such as infrared spectrophotometry and pyrolysis-gas liquid chromatography may also be used to identify man-made fibers (Gaudette, 1988).

Many clothing fibers when found have been exposed to the environment. Important questions that arise in such a case are whether the fiber can be identified and whether it can be meaningfully compared with fibers from the garment or other textile it is suspected of having come from. The fibers may have been exposed to sunlight or water or buried in various types of soil for indeterminate lengths of time. There have been studies on the decomposition of textile fibers, mainly cellulose, in soil (Waksman and Starkey, 1950; Alexander, 1964; Preston, 1986; Siu, 1951), but only a few studies have examined the effect this decomposition has on the identification of the textile fibers (Singer and Rowe, 1989; Northrop and Rowe, 1987; Henrissat and Chanzy, 1986; Siu, 1951).

In addition to its utility as a method for identifying polymers, infrared spectroscopy has proven to be one of the most useful techniques for the study of changes in polymers (Davis and Sims, 1983). It permits both qualitative and quantitative studies of polymer degradation: the increases or decreases in the infrared absorption bands characteristic of specific functional groups are indicative of the gain or loss of such groups by a polymer. Furthermore, infrared spectroscopy requires only a small sample and can be readily applied to insoluble, fibrous polymers.

The wavelength-dispersive infrared spectrophotometers used heretofore in polymer degradation studies suffered from one important drawback: their lack of sensitivity hampered the investigation of the early stages of polymer degradation. The recent introduction of Fourier transform infrared (FTIR) spectrophotometers has significantly remedied this deficiency. Because an FTIR spectrophotometer can acquire the entire infrared spectrum of a polymer sample in approximately two seconds, many hundreds of spectra may be obtained from a sample in the time that was previously required to scan the infrared spectrum once with a wavelength-dispersive instrument. The accumulation of hundreds of spectra from a sample results in an substantially increased signal-to-noise ratio. The enhanced signal-to-noise ratio of FTIR spectrophotometers has made possible the coupling of infrared spectrometers with infrared microscopes. Specially designed infrared microscope objectives permit both visible light examination and FTIR analysis of microscopic specimens.

MATERIALS AND METHODS

The acetate and rayon samples examined in this study were obtained in two previously reported burial studies (Northrop and Rowe, 1987; Singer and

Rowe, 1989). In the first burial study, one centimeter square samples of sixteen textiles composed of man-made fibers (one acrylic, five polyester, five polyamide, and five cellulose acetate) were buried in plastic garden pots containing a commercial garden soil (pH 7.5, 29% organic matter). The pots were maintained at 20-28°C and watered on a weekly basis. The textile samples were exhumed on a monthly basis. In the second burial study, six samples of man-made textiles (cellulose acetate, cellulose triacetate, acrylic, nylon, polyester and rayon) were buried in three different types of soil: undisturbed forest soil (pH 3.9, 6% organic content), urban soil (pH 4.0, 4.6% organic content) and agricultural soil (pH 5.7, 3.5% organic content). Two centimeter square samples were cut from the cellulose acetate and triacetate textiles and one centimeter square samples were cut from the other textiles. These samples were then buried in plastic flower pots. The temperature of the pots was maintained at 20-28°C. The pots were watered once a week by filling them to capacity and allowing the water to drain. The buried textile samples were exhumed on a monthly basis.

In both of the previously reported burial studies, the exhumed fibers were examined microscopically for damage and were then subjected to identification procedures such as solubility, polarized light microscopy, dispersion staining microscopy and pyrolysis-gas liquid chromatography. In the burial study conducted by Northrop and Rowe (1987), the cellulose acetate fibers were the only fibers that showed macroscopic and microscopic evidence of degradation. FTIR spectra of fibers from each of the samples of cellulose acetate textile exhumed after one month, five months and nine months of burial were obtained. In the burial study conducted by Singer and Rowe (1989) only the rayon fibers showed macroscopic and microscopic evidence of degradation. FTIR spectra were obtained from fibers from each the samples of rayon textile exhumed after one month, five months and nine months. All fibers were cleaned by gently brushing soil from them; washing the fibers was not considered because of the friable nature of degraded fibers. The use of an infrared microscope permits the analyst to select areas on the fibers that are free of adherent soil or other contamination. The fibers were maintained at ambient temperature and humidity until the FTIR analysis was carried out. The fibers were flattened prior to spectroscopic analysis to minimize the effects of diffraction and nonuniformity of light path through the specimens. Both diffraction effects and lack of a uniform light path have been shown to effect the measured intensities of infrared absorption bands in the infrared spectra of fine fibers (Chase, 1987; Bartick, 1987). Care was also taken to place the aperture of the FTIR spectrometer well away from the edges of the flattened fibers. All infrared spectra were obtained using a Spectra-Tech IR Plan infrared micro-

scope, coupled to a Nicolet 20SXC FTIR spectrometer equipped with a narrow band MCT detector. The resolution of the spectrometer was 4 cm^{-1}. The infrared absorption spectra were computed using Happ-Genzel apodization. Each of the spectra shown was the result of accumulating 512 scans.

RESULTS AND DISCUSSION

The infrared spectra of standard rayon and rayon buried for nine months in forest and urban soils are shown in Figures 1-3, respectively. Although microscopic examination of these fibers provided clear evidence of degradation, all the major and minor absorption bands of rayon appear with very little change in the spectra of the exhumed specimens. The relative intensity of the hydroxylic νO-H at 3401 cm^{-1} compared to the ethereal and hydroxylic νC-O comprising the band lying between 1200 cm^{-1} and 1029 cm^{-1} varied somewhat from sample to sample. This probably reflects the differing moisture content of the samples. The infrared spectrum of standard cellulose acetate is shown in Figure 4, while the infrared spectrum of a representative specimen of cellulose acetate that had been buried nine months is shown in Figure 5. Again, as in the case of rayon, all of the major and minor absorption bands of cellulose acetate appear in the infrared spectrum of the exhumed specimen. However, in three of the exhumed

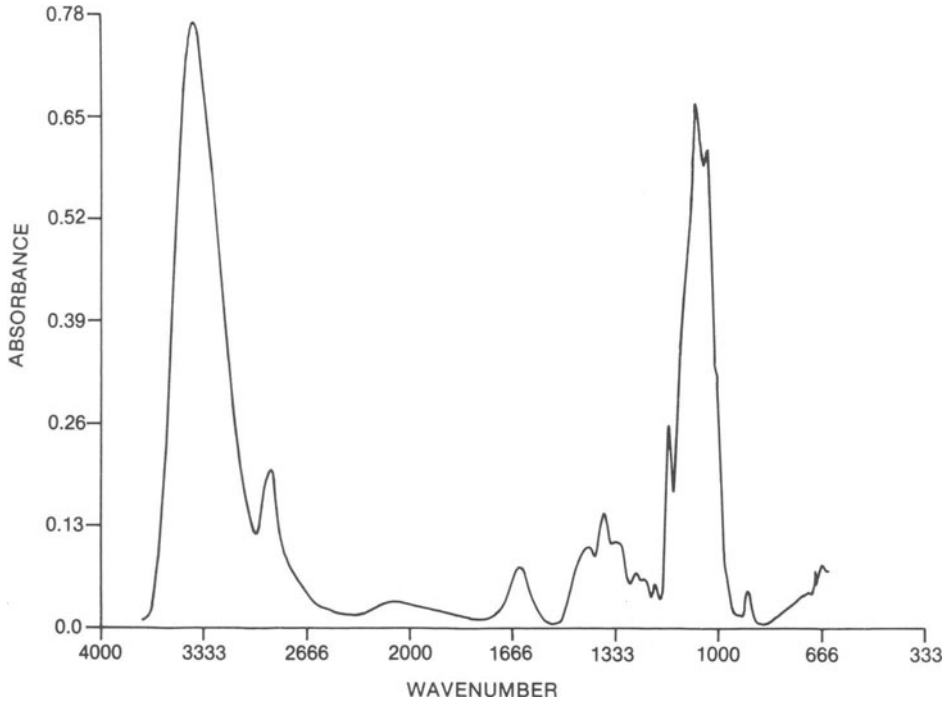

Figure 1. Infrared Spectrum of Standard Rayon.

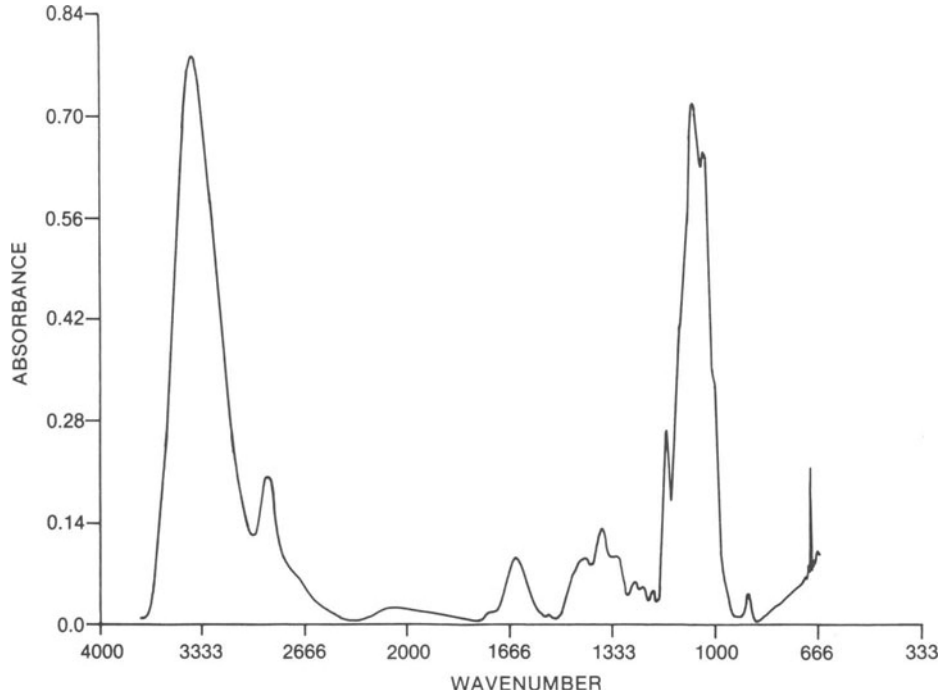

Figure 2. Infrared Spectrum of Rayon Buried Nine Months in Forest Soil.

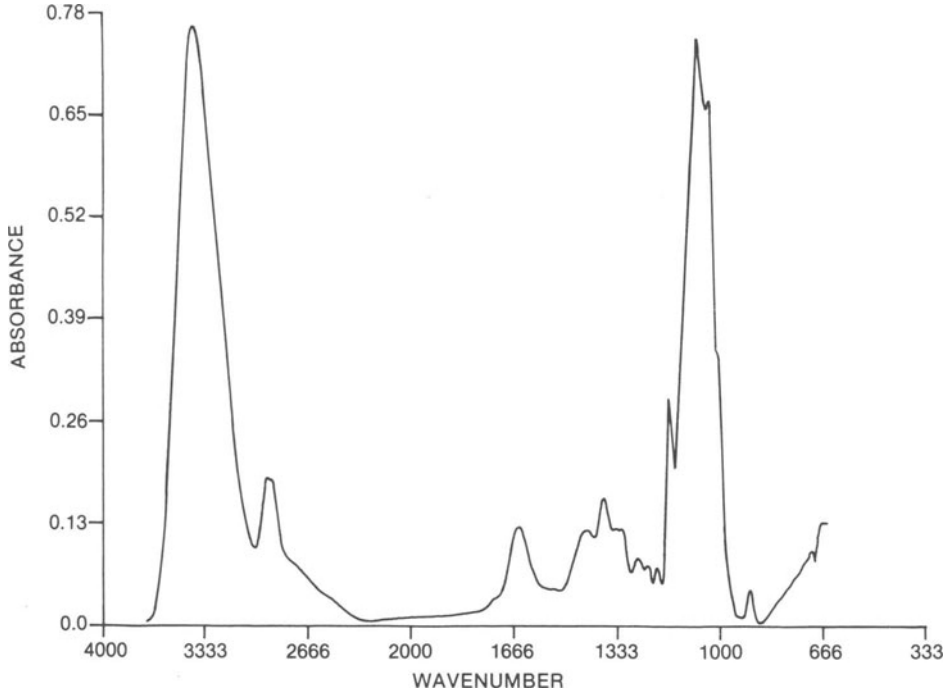

Figure 3. Infrared Spectrum of Rayon Buried Nine Months in Urban Soil.

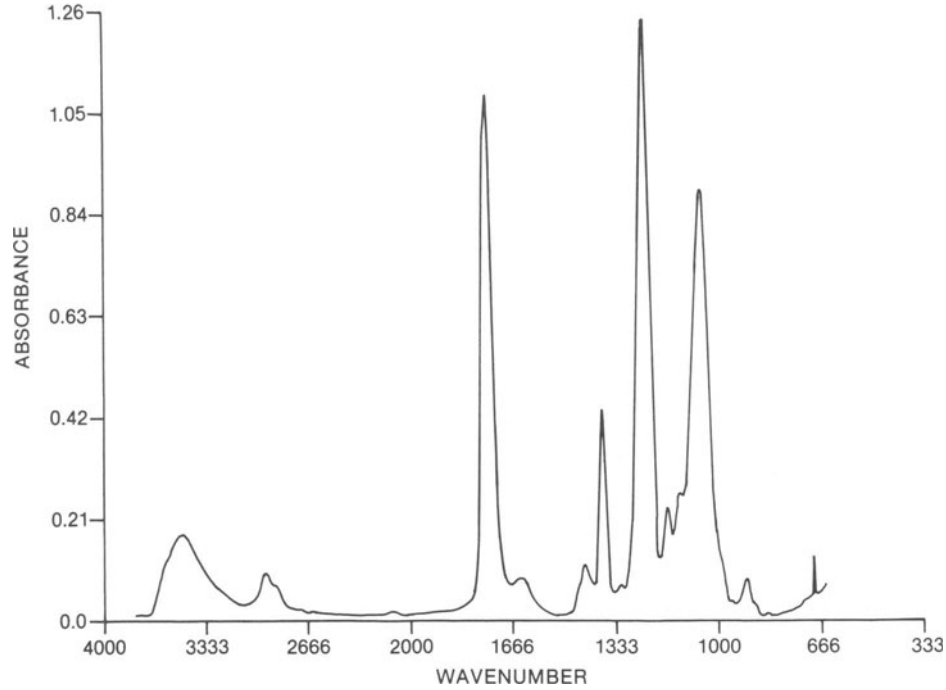

Figure 4. Infrared Spectrum of Standard Cellulose Acetate.

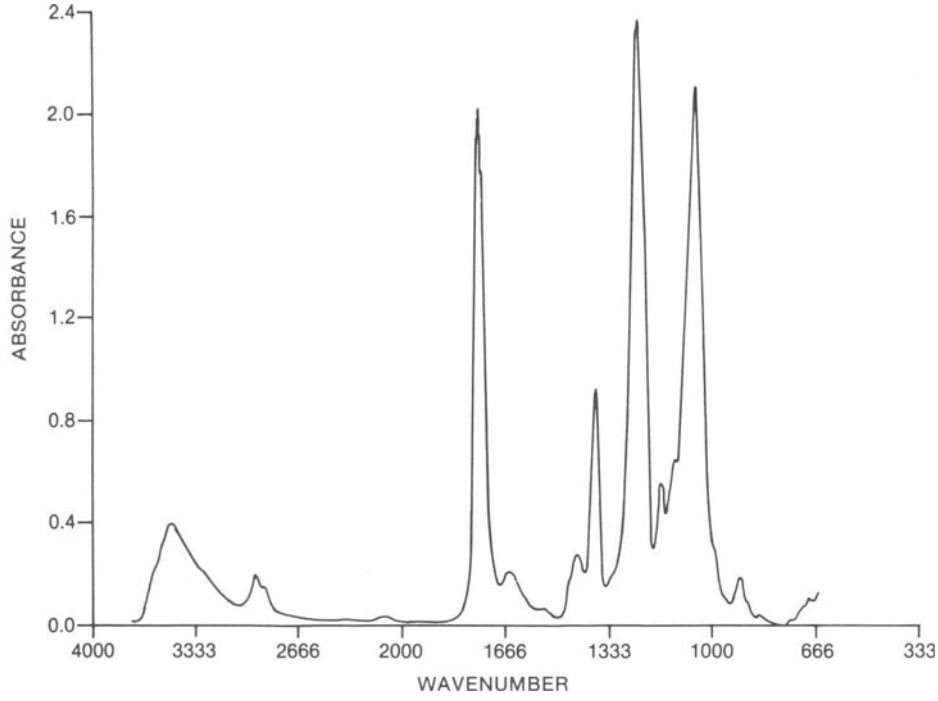

Figure 5. Infrared Spectrum of Cellulose Acetate Buried Nine Months in Commercial Garden Soil.

cellulose samples, substantial decreases in the intensities of the characteristic ester bands at 1747, 1370 and 1236 cm^{-1} relative to the intensity of the band at 1050 cm^{-1} (representing the ethereal and hydroxylic νC-O) were observed. (Small increases and decreases in the relative intensities caused by experimental error in the absorbance measurements were also seen.) The changes in the ratios of the absorbances of the 1747 cm^{-1} and 1236 cm^{-1} ester bands to the absorbance of the 1050 cm^{-1} bands are shown in Figures 6 and 7, respectively, for all of the cellulose acetate samples. These results indicate that the cellulose acetate fibers have undergone partial hydrolysis. Northrop and Rowe (1987) have reported the infrared spectrum of the acetic acid-insoluble residue from degraded cellulose acetate. These workers noted decreases in the intensity of the νC=O band consistent with the loss of acetate groups from the cellulose acetate. It has been noted that many natural and synthetic polymers undergo hydrolytic degradation (Kelen, 1983). Such hydrolytic degradation may be caused either by micro-organisms or by the presence of moisture in the soil.

CASE STUDY

Figure 8 shows the infrared spectrum of a fiber from a fragment of knit textile exhumed during archeological excavations at a historical site near Johnstown, Pennsylvania. The excavators dated the soil deposit in

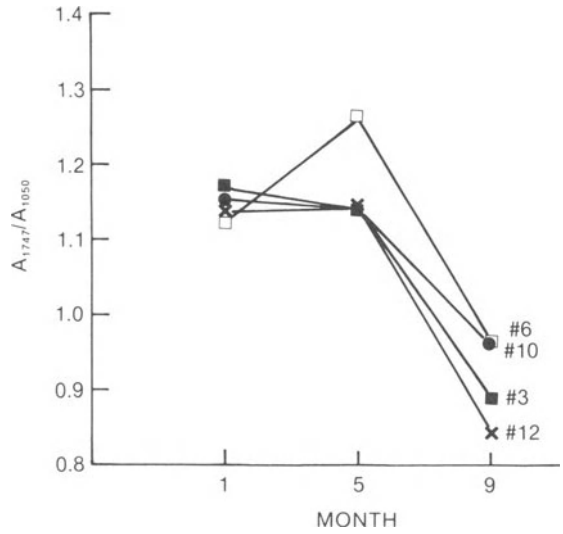

Figure 6. Absorbance Ratios for Infrared Absorbance at 1747 cm^{-1} Versus Infrared Absorbance at 1050 cm^{-1}.

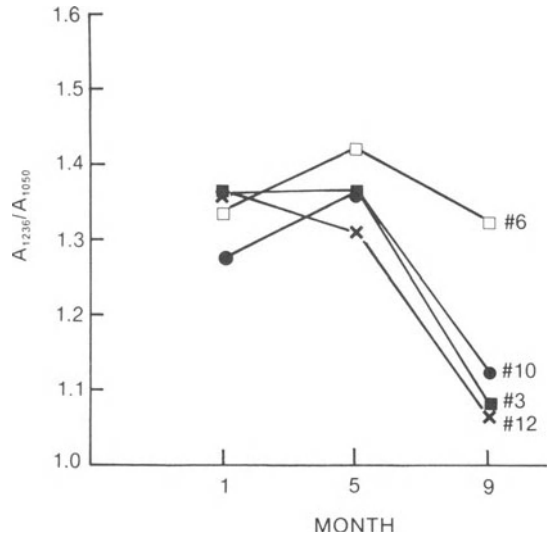

Figure 7. Absorbance Ratios for Infrared Absorbance at 1236 cm^{-1} Versus Infrared Absorbance at 1050 cm^{-1}.

which the textile fragments were found to the 1920s on the basis of historical records. Solubility tests and microscopic examinations indicated that the fibers in the textile were either cellulose acetate or cellulose triacetate. The solubility studies also showed that the fibers had undergone some type of modification in chemical structure: although cellulose acetate fibers are completely soluble in acetone and cellulose triacetate fibers are completely soluble in chloroform (Hollen and Saddler, 1964), these fibers were not completely soluble in either solvent. The formation of insoluble gels as a result of the crosslinking of polymer chains is a feature of the degradation of a number of fibers; however, such crosslinking is almost invariably the result of photo- or thermo-oxidation (Kelen, 1983). Buried fibers are unlikely to have been subjected to either high radiation fluxes or high temperatures; consequently, crosslinking is an untenable explanation for the unusual solubility behavior of these fibers. Figure 9 shows the result of subtracting a standard cellulose triacetate spectrum from that in Figure 8. The absorbances at 3485 cm^{-1} and 1050 cm^{-1} are greater in the spectrum of the exhumed fiber, while those at 1750 cm^{-1} and 1240 cm^{-1} are weaker. These results indicate that the exhumed fiber has fewer acetate groups and more hydroxyl groups than the

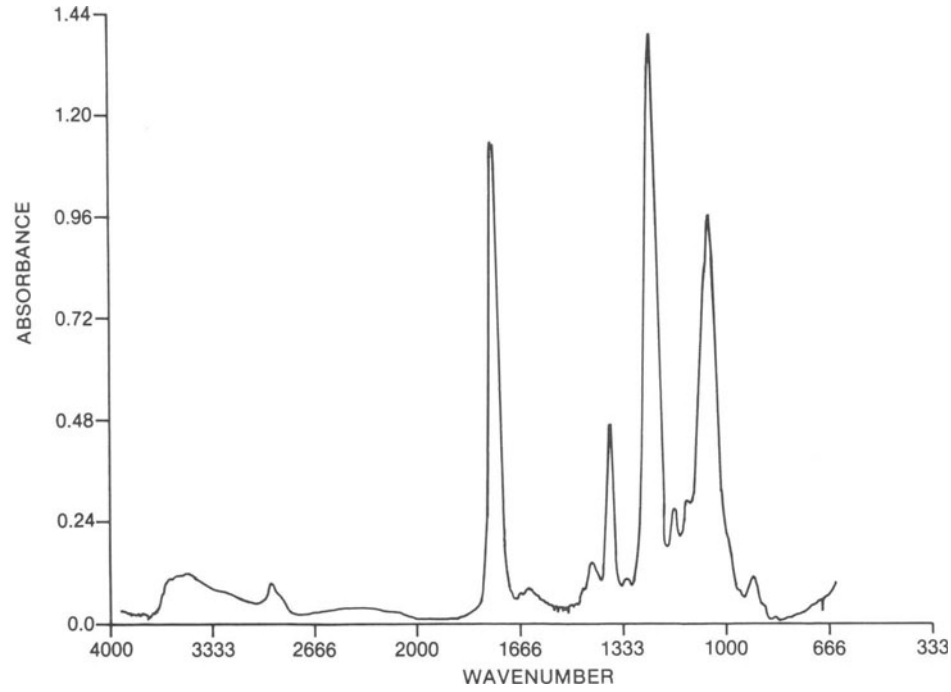

Figure 8. Infrared Spectrum of Suspected Cellulose Triacetate Fiber From Archeological Site Near Johnstown, Pennsylvania.

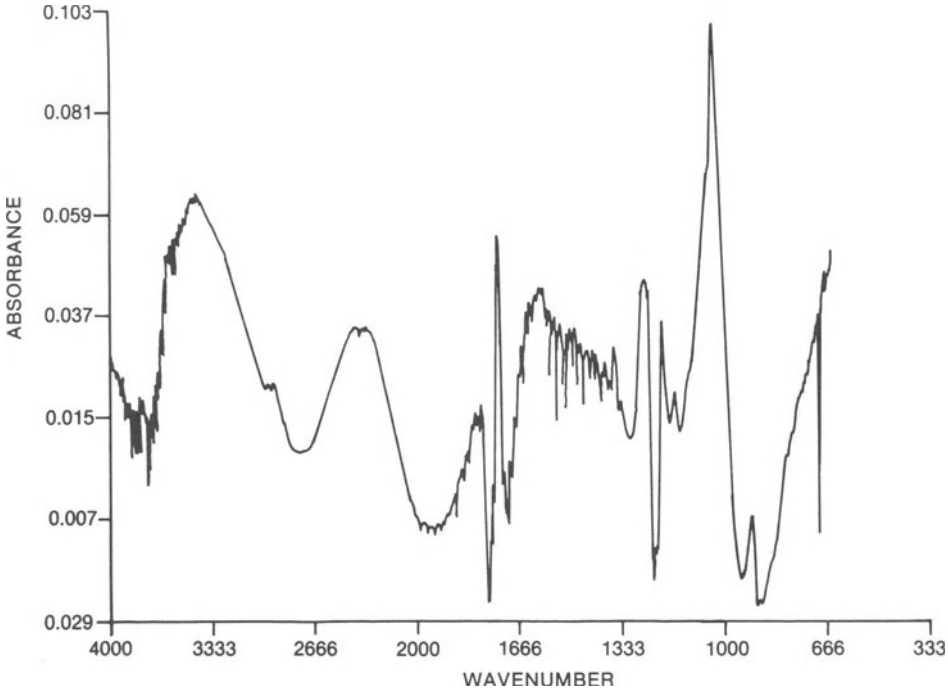

Figure 9. Difference Spectrum Resulting From the Subtraction of Standard Cellulose Acetate Spectrum From Figure 8.

standard cellulose triacetate fiber. At the same time, a comparison of Figure 8 with Figure 4 reveals that the absorbance of the hydroxyl groups at 3485 cm^{-1} is much greater in the spectrum of the standard cellulose acetate fiber than in the spectrum of the exhumed fiber, indicating that the exhumed fiber has more acetate groups and fewer hydroxyl groups than the standard cellulose acetate fiber. Such a compositional difference between the exhumed fiber and the standard cellulose acetate fiber might be ascribed merely to a difference in manufacturing processes. For example, cellulose acetate plastics used in the manufacture of injection molding and extrusion compounds have 2 to 2.5 acetate groups per glucose moiety (Krause et al., 1983). However, cellulose acetate used for fibers has 2.3 acetate groups per glucose moiety (Moncrief, 1975). The spectroscopic and other data for the exhumed fiber are therefore most consistent with a cellulose triacetate fiber that has undergone partial hydrolysis while buried.

SUMMARY

When the infrared spectra of exhumed samples of rayon and cellulose acetate fibers were compared with those of standards, the spectra of the exhumed rayon samples showed only minor differences from that of the standard, while spectra of three of the cellulose acetate samples showed clear evidence of hydrolytic cleavage of the acetate groups. The infrared spectrum of a cellulose triacetate sample from an archeological site also showed evidence of the same type of hydrolytic degradation.

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Factors Involved in Chitin Utilization by Vibrios

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INTRODUCTION

Because cholera remains an infectious disease of public health concern world wide, even with very few cases per year in the United States, the existance of pathogenic vibrios as normal inhabitants in estuarine waters require that we attempt to learn how these organisms maintain themselves in this environment (Colwell et al. 1980). Several investigators have reported that vibrios, although preferring high pH and some salinity, are intimately associated with chitin (Amako et al. 1987; Huq et al. 1984; Guthrie and Cofie, 1987). In those reports the point was made that the presence of organisms such as copepods, as well as larger chitin-bearing organisms, in the water increased the survival time of vibrios which were present.

The purpose of this study was to investigate the effect of chitin or chitin derivatives from several sources on the growth and survival of several strains of Vibrio cholerae, and to attempt to obtain additional evidence supporting the ability of these organisms to maintain themselves in this environment.

MATERIALS AND METHODS

Chitin was extracted from the shells of blue crabs and shrimp from Galveston, Texas bay following drying and pulverizing, according to a modified method (Tracey, 1957). Chitin was also purchased from the Sigma Chemical Company, St. Louis, Mo. as was chitosan and N-acetyl-glucosamine (chitin derivatives).

V. cholerae strains used in this work were 01 strains designated 569B and VC86 from The University of Texas Medical Branch in Galveston; strains 1427, 1271, 1740 and 1968 from Dr. Henry Bradford of the Louisiana State Department of Health; and non-01 strains WF01, CL84 and CL85 isolated from estuarine waters in the Gulf Coast area. All strains were motile, oxidase positive and produced yellow colonies on thiosulfate citrate bile salts sucrose agar (TCBS).

Bay water samples were brought to the laboratory where they were filtered (by negative pressure) through 0.45 um Millipore filters. Filtered water samples were then adjusted to the desired pH, between 6.0 and 9.0 by the addition of 0.1 N NaOH or HCl, and to salinities between 5 and 20 parts per thousand (o/oo) by addition of Instant Ocean Salts when necessary. With these adjustments to test the effects of these parameters on the solubility of chitin, the water was then maintained throughout the tests at a temperature of 22.0 +/- 2.0 C. Total organic carbon (TOC) was measured before and after addition of chitin to a concentration of 0.5 g/L. The measurements after the addition were not done until the elapse of 24 hours to allow solution. The difference between these TOC measurements was considered to be the amount of dissolved chitin in the water.

To study the effect of chitin on growth of the organisms, duplicate microcosms were filled with filtered bay water adjusted to the desired pH and salinity with chitin added to achieve a concentration of 0.5 g/L. To test the growth stimulatory effect of this and other materials, these microcosms were conpared to those to which 0.5% granular peptone, and a 1:10 dilution of sewage effluent were added without the presence of chitin. A control microcosm at each pH and salinity, had no nutrient source added.

Allowing 24 hours for stabilization, each microcosm was then inoculated with approximately 100 cells/ml from a 24 hour culture of V. cholerae in alkaline peptone broth. The cells were washed with buffered saline prior to inoculation of the microcosms to remove the culture broth. At 24 hour intervals following inoculation, each microcosm was sampled and 0.1 ml of the sample was plated to TCBS plates. All microcosms were prepared in duplicate, and all plating was to duplicate plates. Plates were incubated at 35 C for 24 hours before colonies were counted. Each colony was considered to be the product of one colony forming unit.

RESULTS AND DISCUSSION

The tests for chitin solution in bay water samples demonstrated

that salinity was the major factor in determining the solubility of chitin as demonstrated in Figure 1. The influence of pH on the solubility could not be demonstrated. This was found to be the case with chitin from all sources, as well as N-acetyl-D-glucosamine. Chitosan appeared to be less affected by the salinity of the water. A salinity of 15 o/oo was found to be the optimum for solution of chitin in this bay water.

All strains of V. cholerae, both 01 and non-01 were able to use chitin and N-acetyl-D-glucosamine at a concentration of 0.5g/L as is illustrated by growth curves demonstrated in Figures 2-7. Following inoculation of the microcosm with the V. cholerae seed there was daily growth (as indicated by increases in numbers of colony forming units) of all strains tested in alkaline peptone, shrimp chitin, N-acetyl glucosamine, and crab chitin after 24, 48 and 72 hours. This increase continued to a peak at 96 hours in all except N-acetyl-D-glucosamine. In the latter case the peak numbers of cells occurred after 72 hours of growth, and numbers declined thereafter through day 7. The decline in numbers in this chitin derivative was not dissimilar to that observed in alkaline peptone except that it generally began one day earlier.

Chitosan at 0.5 g/L and 10% sewage effluent failed to stimulate growth of any strain of V. cholerae. No strain could be cultured from microcosms containing chitosan for more than 5 days following inoculation, and no strain could be cultured from those containing sewage effluent for more than 4 days. Filtered bay water generally allowed recovery for longer than did these two nutrients.

Although the initial stimulation of growth by alkaline peptone was greater than sources of chitin, in only two cases was this greater cell concentration maintained to the seventh day. The slow decline in counts of organisms from microcosms containing either shrimp shell chitin or crab chitin indicates that the presence of these nutrient sources helps to maintain large populations of V. cholerae in saline waters at these levels of pH and salinity, and may help to explain the maintenance of these organisms as natural inhabitants of estuarine waters.

SUMMARY

The response of nine strains of V. cholerae strains (both 0-1 and non-01) to the presence of different types of chitin in saline waters dependent on salinity than pH, and the growth of these bacteria appeared to be sustained even better than when grown with alkaline peptone as a nutrient source. The ability of these organisms to grow and remain viable with chitin as a sole nutrient source helps to explain how these

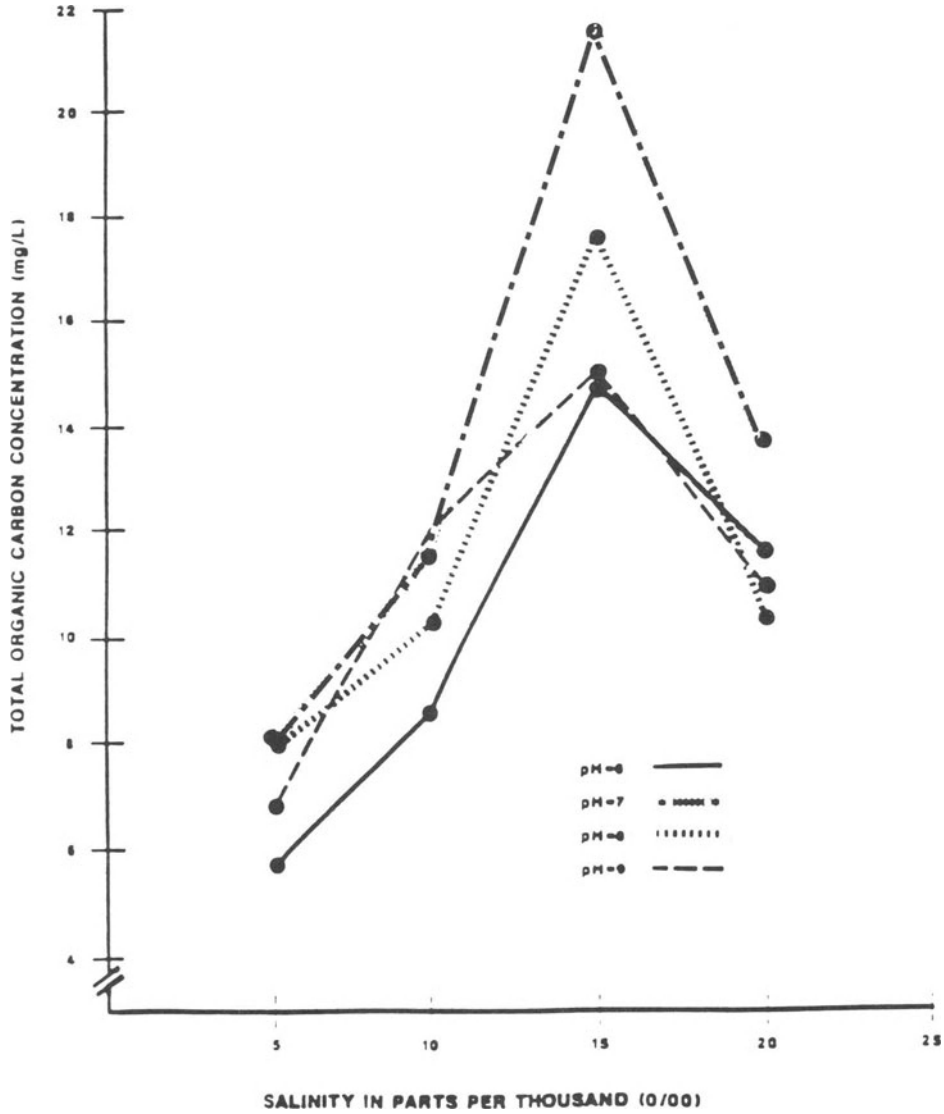

Figure 1. Effect of Salinity and pH on the Solubility of Practical Grade Crab Chitin in Bay Water.

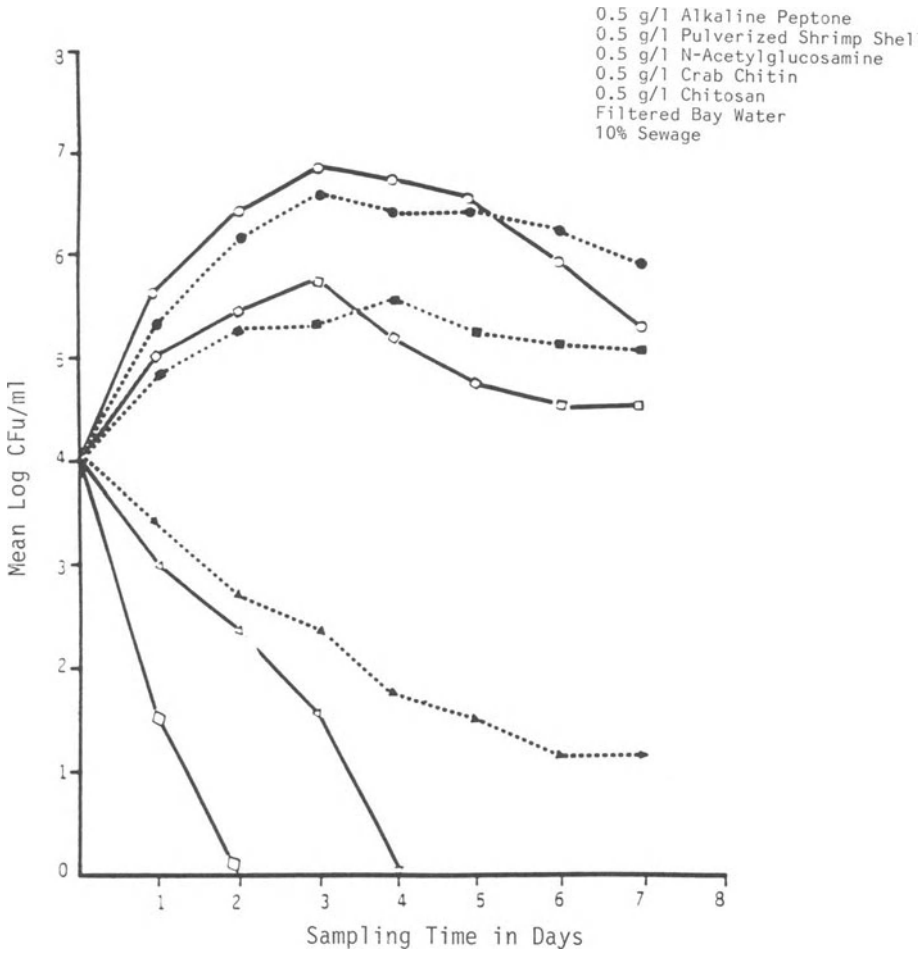

Figure 2. Growth of V. cholerae 569B in Selected Nutrients.

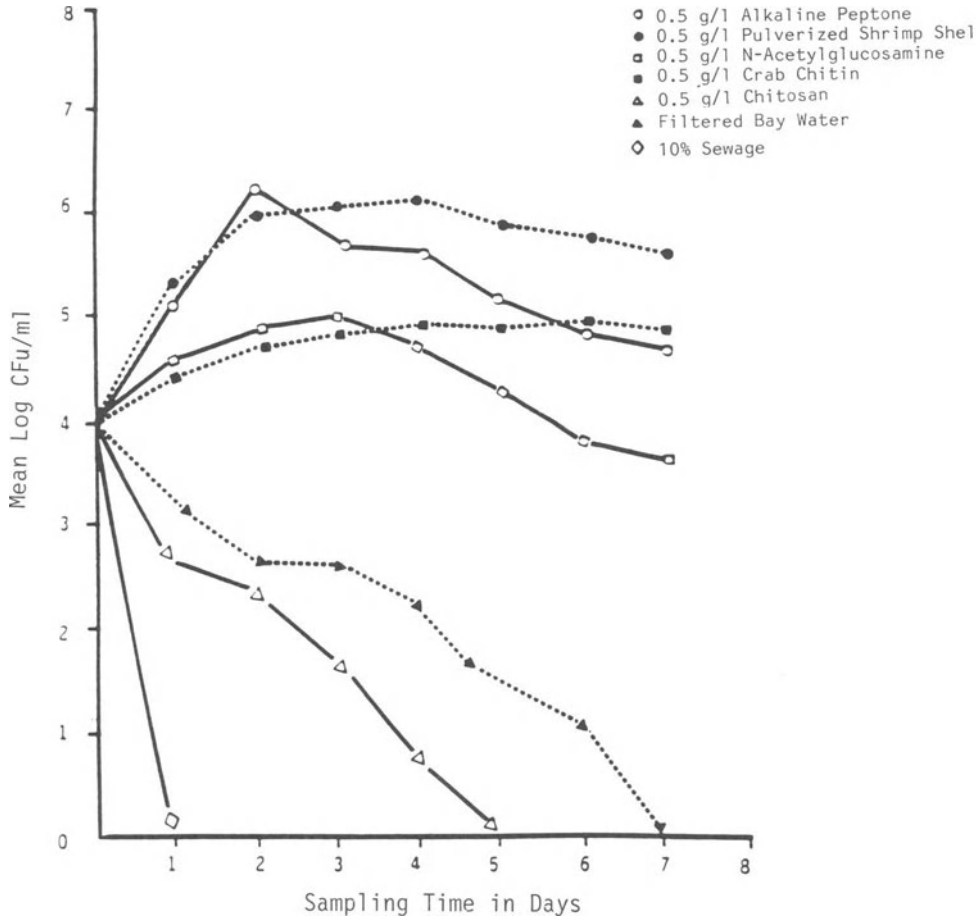

Figure 3. Growth of V. cholerae VC86 in Selected Nutrients.

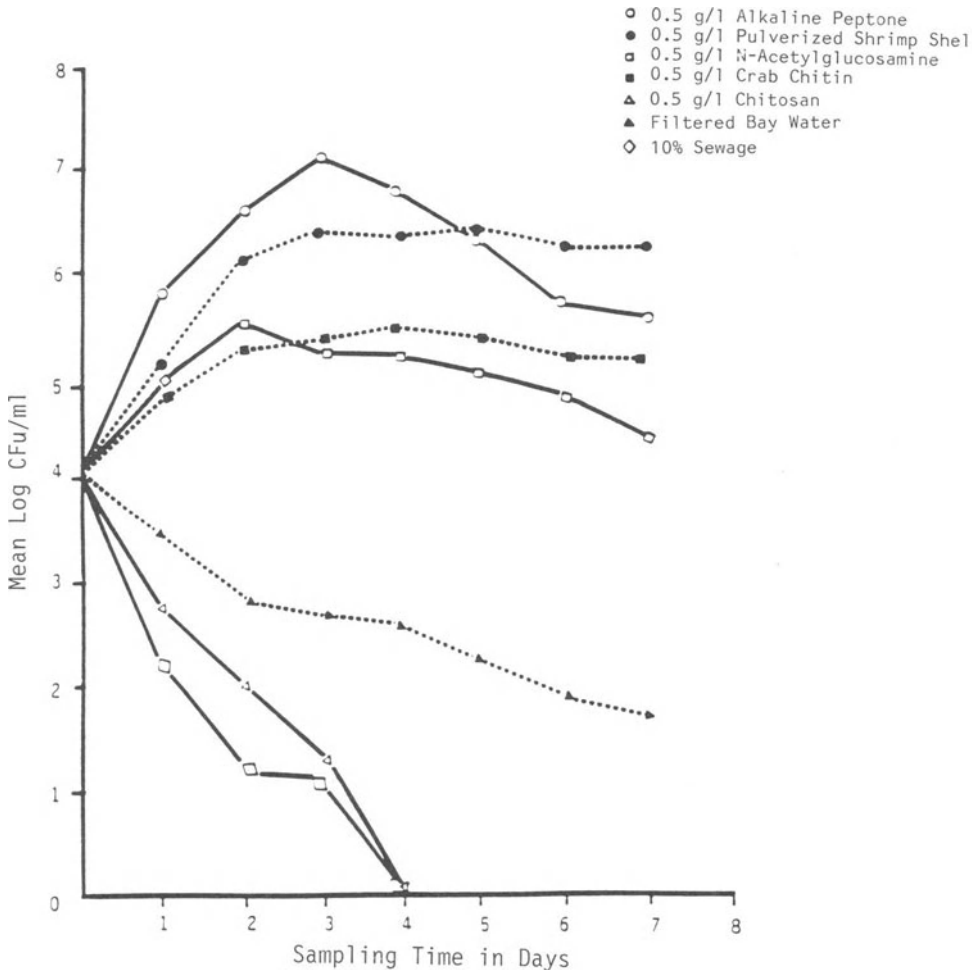

Figure 4. Growth of V. cholerae 1427 in Selected Nutrients.

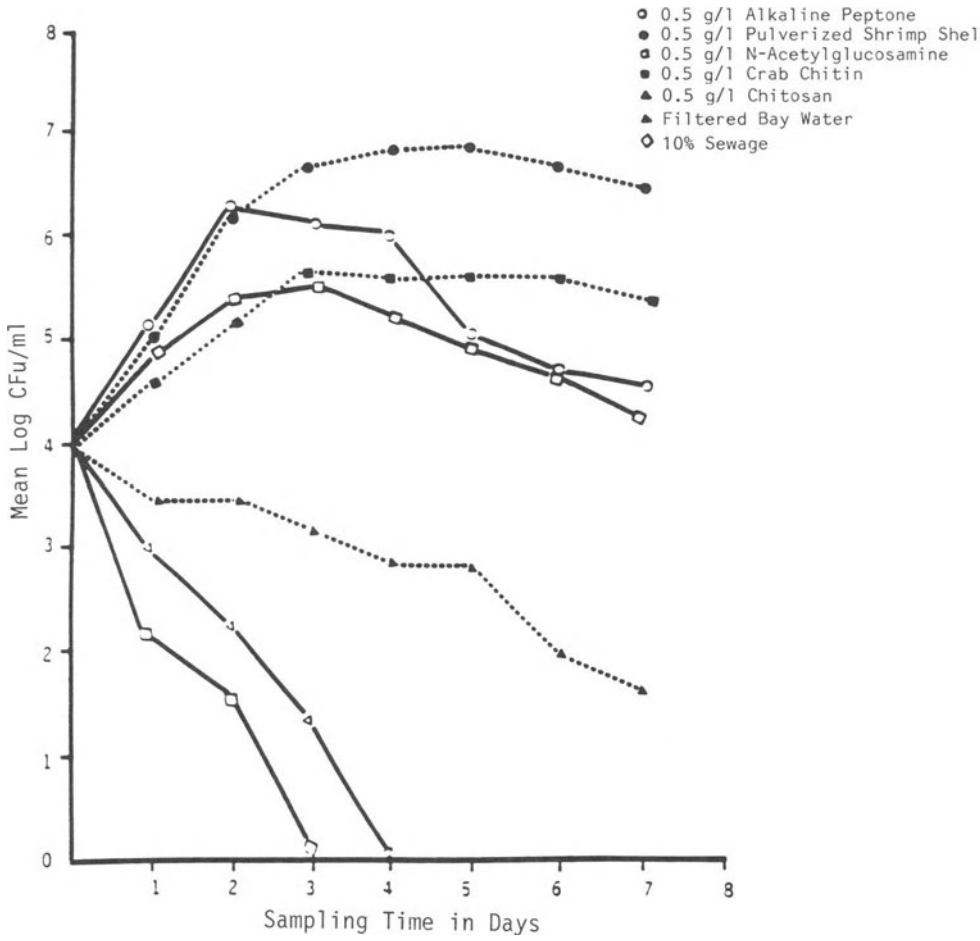

Figure 5. Growth of V. cholerae 1251 in Selected Nutrients.

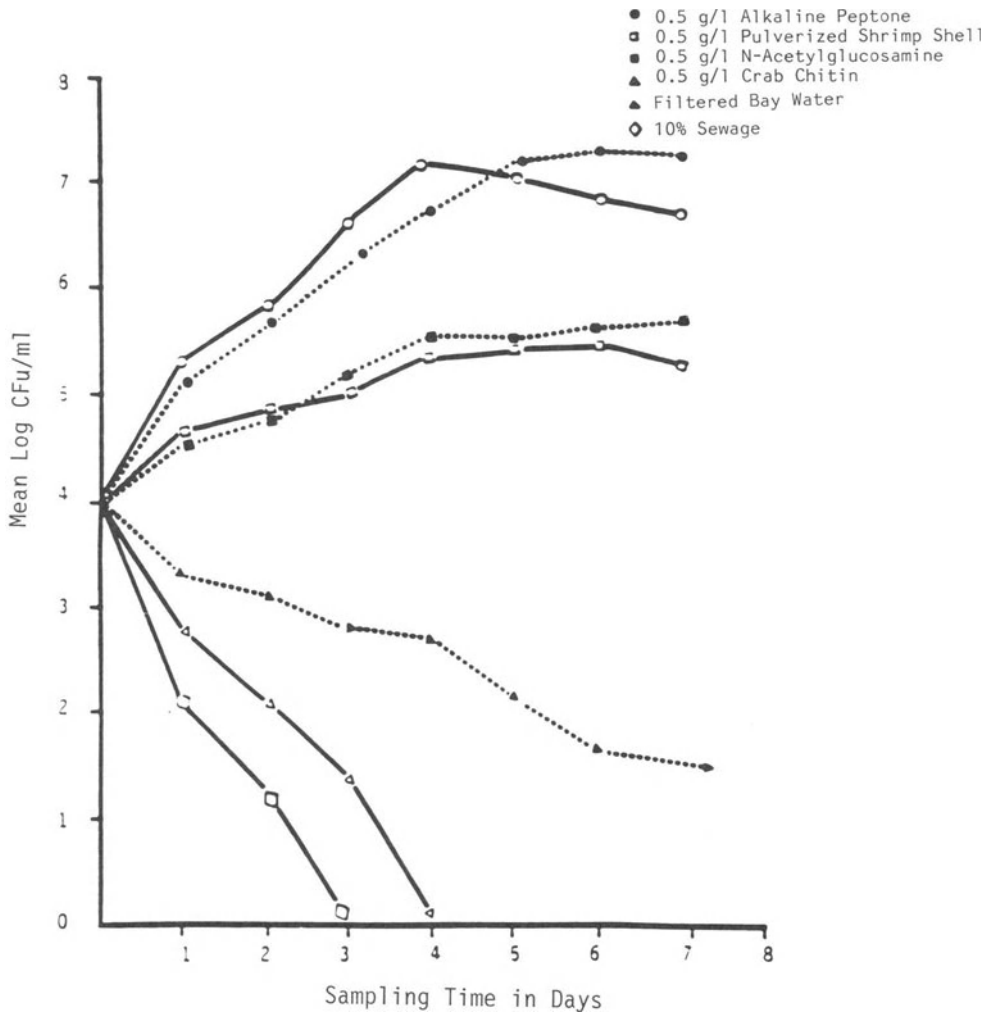

Figure 6. Growth of V. cholerae CL84 in Selected Nutrients.

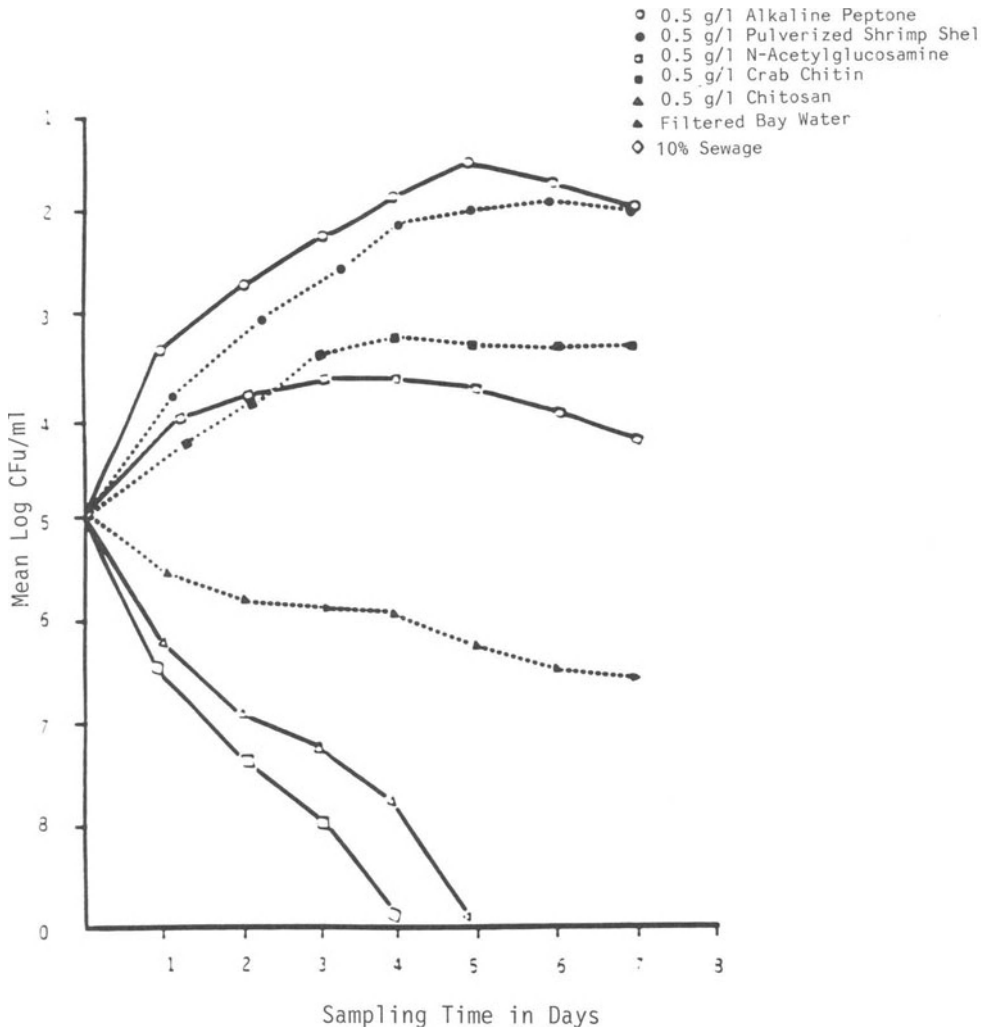

Figure 7. Growth of V. cholerae CL85 in Selected Nutrients.

bacteria are maintained in brackish and estuarine water as natural inhabitants, rather than being brought into these environments carried by domestic sewage wastes.

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Biodegradability Enhancement of Two Xenobiotics in an Industrial Waste as Measured by Respirometry

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INTRODUCTION

Bis(2-chloroethyl)ether (Chlorex or CX) and 2-ethoxyethanol (Cellosolve or CS) were studied earlier to determine the toxicity level of each to the overall function of industrial waste stabilization ponds (Sullivan, 1988; Davis et al, 1989). These three waste stabilization ponds (WSP) operate in series at a major chemicals and plastics industry as follows. A set of primary anaerobic ponds of 28.8 ac (11.72 ha) are followed by a secondary facultative-anaerobic WSP of 153 ac (62.1 ha). That is followed by the tertiary WSP which has 246 ac (100 ha). The detention time through these ponds is 60, 50 and 40 days (150 total), respectively. The flow to the primary WSP is about 0.86 mgd (3.255×10^3 m^3/d) and with other wastewater streams entering the secondary WSP, the final (tertiary) effluent flow is a maximum of 5.04 mgd ($1.91 \times 10^4 m^3/d$). Depths in the ponds are 5-8 ft (1.5-2.4 m) for the primary, 5.6 ft (1.7 m) in the secondary, and 3 ft (0.9 m) in the tertiary WSP. Since the earlier work (Sullivan, 1988) showed the primary WSP influent to have been practically devoid of phosphorus, an investigation was initiated to determine whether the addition of nutrients to the influent would stimulate an oxygen uptake response. Nutrient enhancement of wastewater is not practiced as often as perhaps it should be. Bhargaua et al., (1986) achieved greater B.O.D. removal in WSP's by nutrient supplementation of the wastewater. Paris and Rogers (1986) found that the addition of inorganics to sample waters significantly enhanced the transformation of the organics.

The overall purpose for this research was therefore to measure the B.O.D. response of the three WSP influents to a balanced nutrient ratio and to determine if that nutrient enhancement resulted in increased

organic (including CX and CS) removal. Microbial populations were enumerated because of the aspect of cometabolism which came into consideration during the earlier work.

MATERIALS AND METHODS

The Hach Model 2173 manometric B.O.D. apparatus (Hach Co., 1972) was chosen for this research project. Its larger sample volume, of up to 157 mL, with no dilution streamlines the setup time and reduces the risk of measurement error (Young and Baumann, 1976). In this manner the site-specific microbial populations could be used advantageously (Blok, 1976; Suschka and Ferreira, 1986). Also, this respirometric method lends itself to augmentation with other measurements (C.O.D., T.O.C., and gas chromatography) so that the overall experimental goals could be met (Alsop et al., 1980). By using undiluted WSP influent samples, the B.O.D. responses are more related to field conditions than the standard B.O.D. which contains added seed and nutrients, with only organics being the limiting factor (Sawyer and McCarty, 1978).

The first series of respirometric tests showed little or no response on the B.O.D. scale (Sullivan, 1988). Using past data from the industry for B.O.D., 72 mg/L phosphorus (P) was added to the primary WSP influent samples and 20 mg/L P was added to the secondary and tertiary influent samples. These amounts were calculated to attempt to achieve a B.O.D.:N:P ratio of 100:20:1 in all samples (Gloyna, 1970). Mean values for the primary WSP influent were: T.O.C., 1,952 mg/L (n=44), B.O.D., 2,455 mg/L (n=39), NH_3-N+NO_3-N, 70.05 mg/L, and ortho-phosphate, 0.10 mg/L (n=44). B.O.D.:N:P ratios for the three WSP influents were as follows: primary, 100:2.82:0.004; secondary, 100:9.09:0.015; and, tertiary, 100:43:0.042. Ethylene glycol is manufactured in the plant and was added in the enhancement matrix selectively due to its ease in degradation and use as a cometabolite (Pitter, 1976). It was added at a level to increase the B.O.D. of the sample by 10%, knowing that 1 mg ethylene glycol (EG) = 0.48 mg/L B.O.D.

Total organic carbon (T.O.C.) was measured by the combustion infrared method 505A (APHA et al., 1985). C.O.D. was analyzed using the Hach reactor digestion apparatus. Ammonia was analyzed by the nesslerization method, nitrate by the cadmium reduction method, orthophosphate by the amino acid method (Hach Co., 1984) and standard late counts by method 910A (APHA et al., 1985). Cellosolve (CS) and Chlorex (CX) were quantified by gas chromatography using direct aqueous injection in a Perkin Elmer model 910 GC with a polyethylene glycol 30m

megabore capillary column and FID. The nutrient supplementation matrix of phosphorus (P), ammonia-nitrogen (N) and ethylene glycol (EG) was P, N, EG, P+N, P-EG, N-EG and, P+N+EG. For instances when the manometric B.O.D. readings approached the scale maximum of 350 mg/L, the unit was opened, purged with air, resealed and continued from zero setting to obtain cumulative values.

RESULTS AND DISCUSSION

Preliminary runs of primary, secondary and tertiary influent nutrient supplementation (Tables 1, 2, 3) showed dramatic oxygen uptake increases in every sample which had phosphorus added. The oxygen uptake values in those tables are shown as B.O.D.5 values. Overall lower values in the secondary and tertiary pond influent samples were due to the reduced organic content by treatment in the ponds. Some stimulation of activity in the primary influent (Table 1) is shown for the addition of EG alone. That was not shown as readily in the secondary and tertiary influent samples. The greatest T.O.C. and C.O.D. reductions shown in Table 1 were in the samples which had the phosphorus added. The secondary pond (Table 2) samples with phosphorus additions showed the highest percentage reductions. It should be noted that the waste strength was roughly one-third that of the primary influent. By the time the wastewater reaches the tertiary pond influent (Table 3) the T.O.C. was only 35 mg/L and the C.O.D. was 95 mg/L. Reduction of organics at day 5 was evident as was stimulation of oxygen uptake by phosphorus addition.

Ethylene glycol additions as stated earlier were for the purpose of attempting to stimulate degradation of possible recalcitrant organics by cometabolism. This phenomenon has been described as a slow conversion of compounds without an increase in microbial biomass or number of bacteria (Richards and Shieh, 1986). More often than not one species of bacteria may not degrade an organic but two or more species can. Another description of cometabolism deals with our reason for adding ethylene glycol. The addition of an easily degradable organic significantly enhances the degradation of the target organics (Alexander, 1981; Richards and Shieh, 1986; Petrasek et al., 1983; Tabak et al., 1981). Respiration rates with viable microbial populations apparently are not constant. Increases have been reported (Walker and Davies, 1977) at very low growth rates. This too could be cometabolism. In a complex wastewater such as the one in this project the likelihood of only one mechanism contributing to the overall degradation of the

Table 1. Primary Pond Influent (7/21/87) Responses to Selected Nutrient Additions.

Sample Treated	BOD5	T.O.C., mg/L Day 0/5	T.O.C., mg/L % Reduction	C.O.D., mg/L Day 0/5	C.O.D., mg/L % Reduction
Control	50	840/843	+0.3	2717/2597	4
P	675	840/592	29	2717/1552	42
N	43	840/823	2	2717/2380	12
EG	118	923/932	+0.8	2995/2618	12
N+EG	73	923/924	+0.1	2995/2576	14
P+EG	567	923/706	23	2995/1852	38
P+N	678	840/556	33	2717/1548	43
P+N+EG	730	923/606	34	2995/1620	45

Table 2. Secondary Pond Influent (5/26/87) Responses to Selected Nutrient Additions.

Sample Treated	BOD5	T.O.C., mg/L Day 0/5	T.O.C., mg/L % Reduction	C.O.D., mg/L Day 0/5	C.O.D., mg/L % Reduction
Control	36	296/192	35	816/463	43
P	430	296/40	86	816/83	89
N	60	296/175	40	816/417	48
EG	39	324/202	37	908/435	52
N+EG	32	324/202	37	908/444	51
P+EG	378	324/43	86	908/81	91
P+N	414	296/41	86	816/61	92
P+N+EG	402	324/44	86	908/94	89

Table 3. Tertiary Pond Influent (7/28/87) Responses to Selected Nutrient Additions.

Sample Treated	BOD5	T.O.C., mg/L Day 0/5	T.O.C., mg/L % Reduction	C.O.D., mg/L Day 0/5	C.O.D., mg/L % Reduction
Control	5	35/30	14	95/78	17
P	46	35/26	25	95/66	30
N	7	35/34	2	95/79	16
EG	8	49/43	12	141/111	21
N+EG	8	49/44	9	141/105	26
P+EG	38	49/25	49	141/58	58
P+N	18	35/30	14	95/76	20
P+N+EG	55	49/24	50	141/71	49

organics is remote. Indeed, as stated by Guthrie (1988), it consists of a combination of digestion or mineralization of complex naturally occurring or synthetic molecules, or combinations of simpler molecules. These points should be kept in mind when examining the data.

The second set of analyses was conducted for the same purposes but with more specific direction. Variability of quality in the wastewaters was determined, bacteria were enumerated and Cellosolve (CS) and Chlorex (CS) were quantified. Figures 1 and 2 and Table 4 contain the data for two runs on different dates of the primary influent. In all of these runs, CS was added at a concentration of 50 mg/L and CX was added at 10 mg/L. Those samples which had phosphorus added showed early increases in oxygen uptake responses compared to those without phosphorus (Figures 1, 2). At day 3 of the 5-day test, most of those responses tended to plateau. There was a significantly greater decrease in T.O.C. for the 9/13/88 sample (Table 4) than for the 11/3/88 sample. Samples with phosphate tended to have higher T.O.C. reductions but it was not as consistent as in the first set (Table 1). Reduction of CS as well as CX was enhanced by nitrogen additions in addition to phosphorus. CX reduction was not clearly related to one particular nutrient nor did it degrade to the significant extent that was reported by Tabak et al., (1981). They found that 10 mg/L CX degraded to 100% in 7 days by using 1-3 microbial subcultures. Microbial populations (Table 4) showed distinct increases at day 5 compared to day 0, shown in parenthesis. Yet, there were no consistent correlations with those data sets which showed the highest degradation rates.

The first run on the secondary pond influent (Figure 3) indicated that the different nutrient treatments did not have as marked a response as in the primary pond samples. Note the general clumping of data at day 5. The maximum B.O.D. attained was 178 mg/L. The data in Figure 4, for the second run with secondary influent, appear to have responded similarly to the trend for the primary influent. The maximum B.O.D attained in Figure 4 was 321 mg/L. Data for these 2 runs show the high temporal variability of the waste streams. Table 5 contains the remaining analytical data for the 2 secondary influent runs shown in Figures 3 and 4. Note that the data in Table 5 for 10/18/88 has a reduced nutrient matrix. That sample contained a high concentration of ammonia: therefore none was added. It also contained a higher organic concentration (168 mg/L vs 71 mg/L for the 9/20/88 sample). Percentage reductions of T.O.C. were not greatly different between dates. The activity in the secondary pond influent samples was significantly

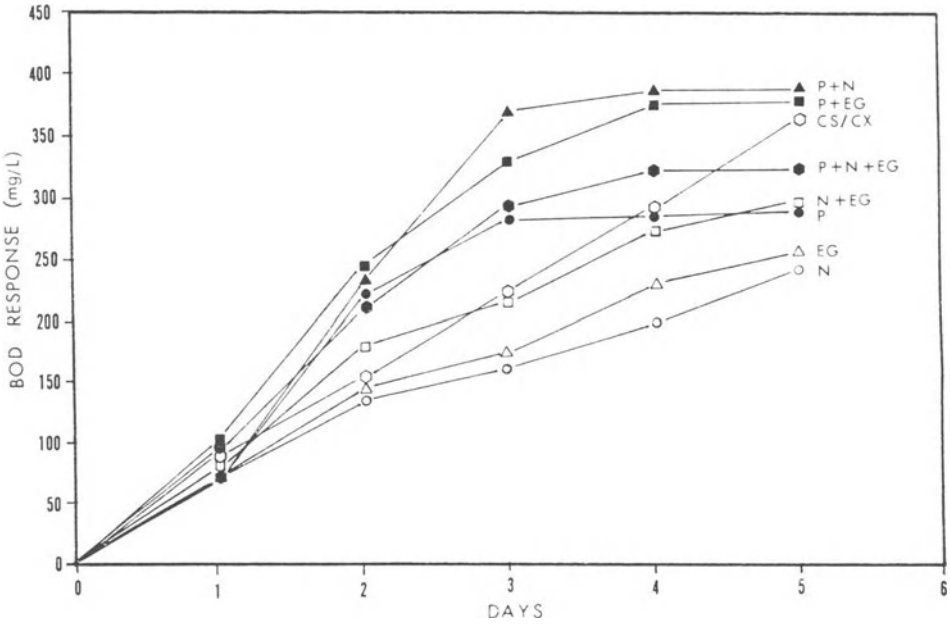

Figure 1. Respirometric B.O.D. Responses of Primary Pond (1°ry) Influent (9/13/88) for Selected Nutrient Conditions.

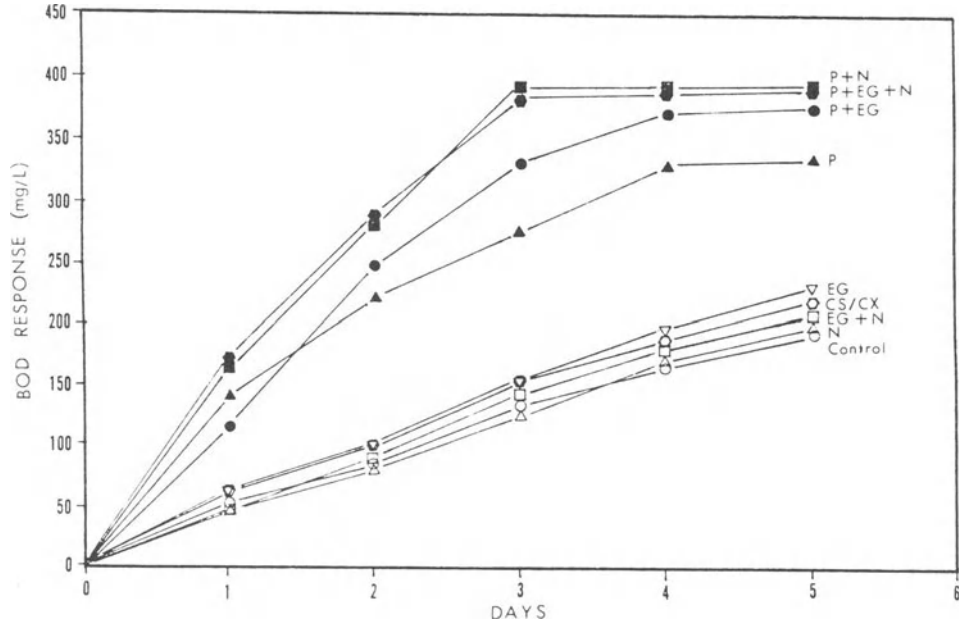

Figure 2. Respirometric B.O.D. Responses of Primary Pond (1°ry) Influent (11/3/88) for Selected Nutrient Conditions.

Table 4. Primary Pond Influent Responses to Selected Nutrient Additions and Bacteria Counts.

Sample Treated	T.O.C., mg/L Day 0/5	% Redn.	Cellosolve, mg/L Day 0/5	% Redn.	Chlorex, mg/L Day 0/5	% Redn.	Log CFU
Sample Date 9/13/88							
1°ry Infl.	1500/-	--	N.D./-	--	N.D./-	--	(6.50)
CS/CX	1530/905	41	50/42	16	10/8.2	18	8.41
P	1530/1030	33	50/28	44	10/8.2	18	8.11
N	1530/1099	28	50/13	74	10/8.1	19	8.25
EG	3064/2213	28	50/42	16	10/9.1	9	8.30
P+N	1530/917	40	50/35	30	10/6.7	33	8.18
P+EG	3064/2018	34	50/44	12	10/8.1	18	7.18
N+EG	3064/2127	31	50/27	46	10/6.5	35	8.56
P+N+EG	3064/2093	32	50/29	42	10/8.3	17	8.43
Sample Date 11/1/88							
							(7.11)
1°ry Infl.	2162/1983	8	N.D.	--	17/12	29	10.04
CS/CX	2191/2005	8	50/20	59	27/22	19	>9.75
P	2191/1787	18	50/18	63	27/29	0	>11.75
N	2191/2042	7	50/33	35	27/28	0	9.86
EG	2417/2160	11	50/33	35	27/23	16	9.34
P+N	2191/1709	22	50/22	57	27/20	25	13.11
P+EG	2417/2041	29	50/21	57	27/27	2	11.68
N+EG	2417/2209	9	50/33	34	27/26	6	10.50
P+N+EG	2417/2013	17	50/21	57	27/28	0	13.04

N.D. = None Detected

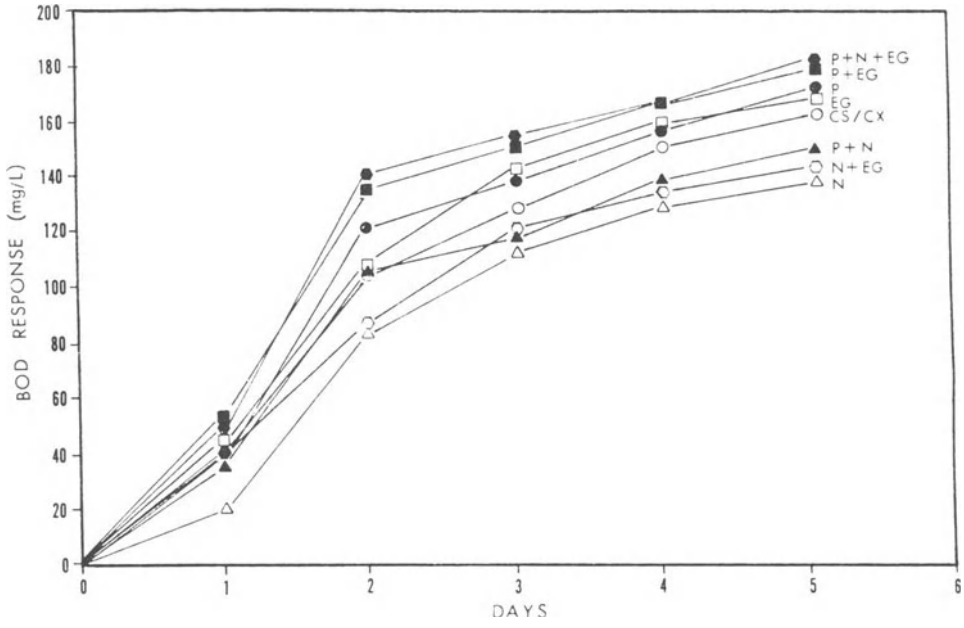

Figure 3. Respirometric B.O.D. Responses of Secondary Pond (2°ry) Influent (9/20/88) for Selected Nutrient Conditions.

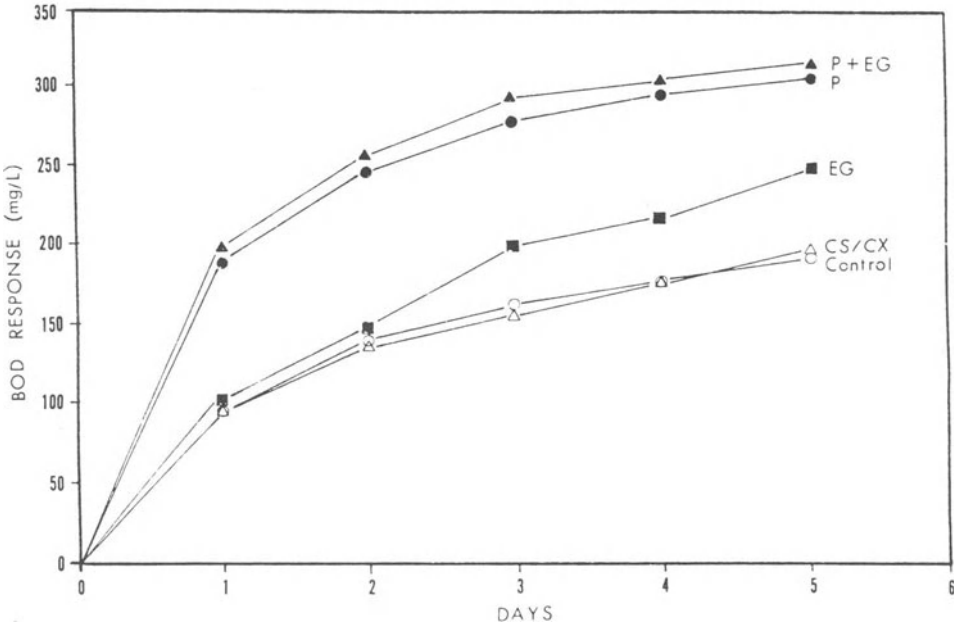

Figure 4. Respirometric B.O.D. Responses of Secondary Pond (2°ry) Influent (10/18/88) for Selected Nutrient Conditions.

Table 5. Secondary Pond Influent Responses to Selected Nutrient Additions and Bacteria Counts.

Sample Treated	T.O.C., mg/L		Cellosolve, mg/L		Chlorex, mg/L		Log CFU
	Day 0/5	% Redn.	Day 0/5	% Redn.	Day 0/5	% Redn.	
Sample Date: 9/20/88							
2°ry Infl.	71/-	--	N.D./-	--	1.6/-	--	(9.30)
CS/CX	101/-	--	50/3.1	94	11.6/7.1	39	9.07
P	101/35	66	50/2.7	95	11.6/7.5	35	9.20
N	101/37	63	50/2.8	94	11.6/6.3	46	9.38
EG	111/32	71	50/2.8	94	11.6/7.4	36	9.60
P+N	101/28	72	50/2.1	96	11.6/4.7	60	9.45
P+EG	111/33	71	50/2.4	95	11.6/7.0	40	9.67
N+EG	111/28	75	50/3.2	94	11.6/9.4	19	9.54
P+N+EG	111/30	73	50/3.0	94	11.6/10.3	11	9.04
Sample Date: 10/18/88							
							(9.95)
2°ry Infl.	168/41	76	N.D.,/N.D.	--	1.2/N.D.	≈100	9.96
CS/CX	62/19	69	50/1.8	96	11.2/7.6	72	9.88
P	198/39	80	50/0.5	99	11.2/5.5	51	9.00
EG	217/70	68	50/1.9	96	11.2/7.6	72	9.61
P+EG	217/36	83	50/0.2	99	11.2/4.6	59	9.23

N.D. = None Detected

different for both CS and CX reduction. Much higher reduction amounts occurred in both secondary influent samples. Interestingly enough, the bacterial populations did not change in numbers for either secondary influent sample from day 0 to day 5. Recall the cometabolism discussion above.

The tertiary influent B.O.D. responses varied between samples (Figures 5, 6). This influent has a much lower organic concentration (Table 6) but the responses to nutrient additions was as dramatic as in the primary influent. Nitrogen was not added to either tertiary sample because ammonia was present in adequate amounts. At this point in the WSP scheme the bacterial populations are well established and acclimated. The B.O.D. responses for nutrient additions did not compare to the T.O.C. reduction values (Table 5) in all cases. While significant reductions are shown over the controls there does not appear to be any one nutrient that was responsible for a greater reduction. CS was significantly reduced in both tertiary influent samples. CX on the other hand was not reduced nearly as much as in the secondary influent samples. For unknown reasons CS was not reduced significantly in the 10/25/88 tertiary sample. There were no significant differences in bacterial populations from day 0 to day 5 in either tertiary influent sample (Table 6). Nor was there any outstanding increase (or decrease) in concentration of bacteria between treatments of chemicals. Again, a cometabolism response may have been what these data reflect.

SUMMARY

Influent samples to three industrial waste stabilization ponds (WSPs) which are operated in series were investigated to evaluate the oxygen uptake (as B.O.D.) responses after adding inorganic nutrients to a ratio of near 100:20:1 as B.O.D.:N:P. Oxygen uptake responses were compared to the amounts and percentages of Cellosolve and Chlorex which were degraded.

In the primary pond first series, excellent B.O.D. increases occurred in samples to which phosphorus had been supplemented. T.O.C. and C.O.D. were reduced by up to 34% and 45%, respectively. The secondary WSP influent also showed excellent B.O.D. increases with phosphorus supplementation. And, the T.O.C. and C.O.D. reductions were up to 86% and 92%, respectively. The tertiary pond influent, by nature of treatment has a much lower organic content. Nevertheless, phosphorus additions resulted in increased B.O.D. responses with corresponding T.O.C. and C.O.D. reduction of up to 50% and 58%, respectively.

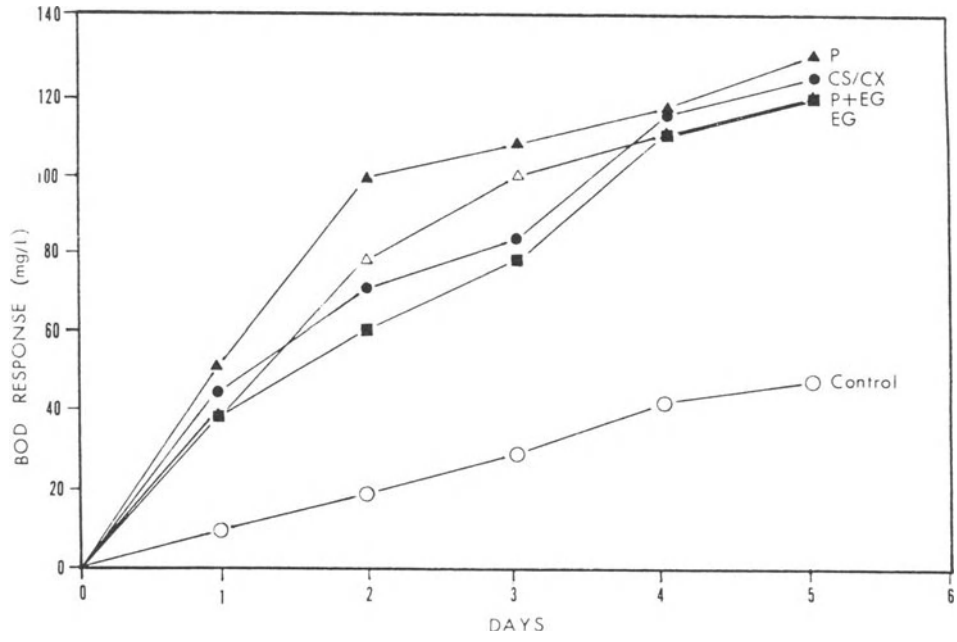

Figure 5. Respirometric B.O.D. Responses of Tertiary Pond (3°ry) Influent (9/27/88) for Selected Nutrient Conditions.

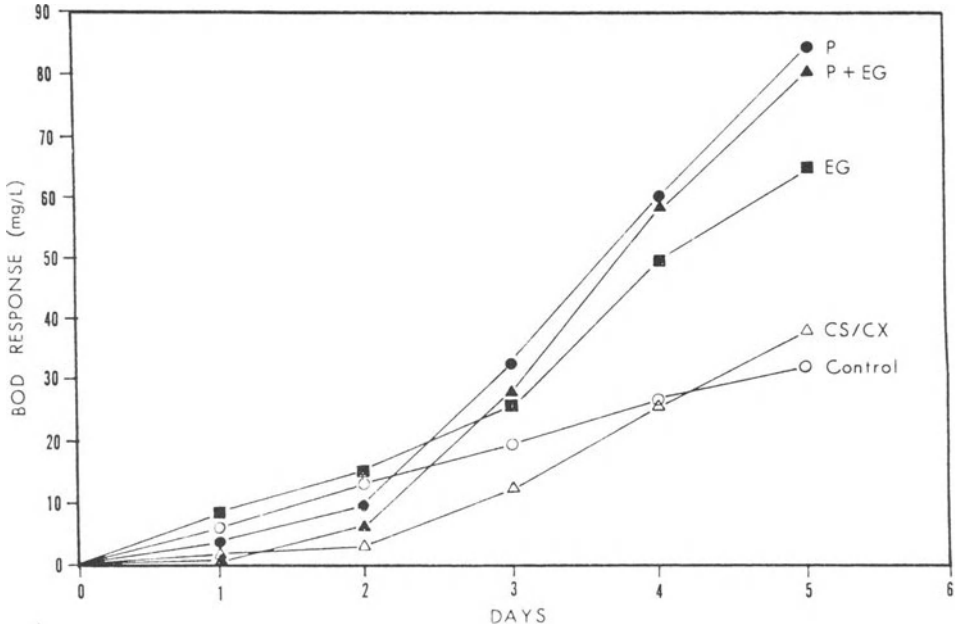

Figure 6. Respirometric B.O.D. Responses of Tertiary Pond (3°ry) Influent (10/25/88) for Selected Nutrient Conditions.

Table 6. Tertiary Pond Influent Responses to Selected Nutrient Additions and Bacteria Counts.

Sample Treated	T.O.C., mg/L		Cellosolve, mg/L		Chlorex, mg/L		Log CFU
	Day 0/5	% Redn.	Day 0/5	% Redn.	Day 0/5	% Redn.	
Sample Date: 9/27/88							
							(6.60)
3°ry Infl.	42/25	40	N.D./N.D.	--	N.D./N.D.	--	6.77
CS/CX	72/38	47	50/1.4	97	10/6.3	37	7.08
P	72/44	39	50/2.0	96	10/6.8	32	6.55
EG	74/35	53	50/1.6	97	10/7.5	25	7.04
P+EG	74/34	54	50/2.0	96	10/7.7	23	7.46
Sample Date: 10/25/88							
							(7.15)
3°ry Infl.	42/25	25	N.D./N.D.	--	N.D./N.D.	--	6.36
CS/CX	72/42	41	50/4.5	91	10/9.6	4	7.34
P	72/44	39	50/4.2	92	10/9.5	5	8.86
EG	74/47	37	50/3.7	93	10/9.0	10	7.41
P+EG	74/40	44	50/2.9	94	10/9.9	1	7.15

N.D. = None Detected

The second series of experiments had the same objectives but analyses were done for Cellosolve (CS), Chlorex (CX), total plate counts, and T.O.C. as well as B.O.D. Primary, secondary and tertiary WSP influent samples from separate dates all showed different responses due to variability in the wastewater contents. Nutrient supplementation for the primary WSP samples showed substantial B.O.D. increases with corresponding decreases in T.O.C. CS had reductions of up to 44% and CX had reductions up to 35%. Bacterial populations increased significantly of up to or more than two orders of magnitude.

Secondary WSP influent responses to nutrient additions showed T.O.C. reductions of up to $<73\%$ with $\geq 94\%$ CS reduction and $<72\%$ CS reduction. Bacterial populations did not increase at day 5 as they did in the primary WSP runs. A strong indication of cometabolic microbial activity was shown here. A similar bacterial population response was shown in the tertiary WSP samples. T.O.C. reductions varied between nutrient treatments with the maximum reduction being 54%. CS and CX reductions were $>90\%$ and from 1-37%, respectively. Investigation of oxygen uptake responses by respirometric methods can be applied to any wastewater when the objective is one of increasing the efficiency of operation for overall reduction of environmental contamination.

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The Effects of Temperature, Nutrients, and Spore Concentration on the Germination of Conidia from *Dactylomyces thermophilus*

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INTRODUCTION

Cooney and Emerson (1964) defined thermophilic fungi as those fungi having a minimum temperature for growth at or above 20C and whose maximum temperature for growth is at or above 50C. Thermophiles have been isolated from a wide variety of habitats and contribute to the decomposition of materials such as stored products, municipal wastes, and compost. Recently, Deploey and Gautam (1987), and Deploey (1989) reported results of studies concerning the germination of spores from two thermophilic species of Rhizomucor (R. miehei and R. pusillus). Other studies of the germination of spores from thermophilic and thermotolerant fungi have also been reported (Celerin and Fergus, 1971; Deploey, 1985; Fergus and Delwiche, 1975; Jack and Tansey, 1977; Streets and Ingle, 1972; Sussman, 1976), but of these only one (Jack and Tansey, 1977) concerned the germination of spores from Dactylomyces thermophilus. In their study Jack and Tansey (1977) reported the results of germination studies of D. thermophilus conidia exposed to sun-heated soil, sun-shaded soil, and at 22-24C and 37C in the laboratory. However, their study of D. thermophilus did not include any other parameters influencing spore germination. Since so little is known concerning the germination of conidia from D. thermophilus, studies were done here to determine the effects of temperature, spore concentration, and nutrients on the germination of these spores.

MATERIALS AND METHODS

The thermophilic fungus Dactylomyces thermophilus (isolate NRRL 5208), was obtained from the Department of Biology of The Pennsylvania

State University. The fungus was grown in tubes containing slanted glucose-yeast extract agar (Adams, 1981). A conidial suspension was obtained from four-day-old cultures, and the spores were washed using a method previously described (Deploey, 1985). A Fuchs-Rosenthal counting chamber was used to count the spores, and their concentration was adjusted to 500,000/ml (except where indicated). In the two temperature studies, and in the study of the effects of spore concentration on germination, a defined glucose-basal (GB) agar contained in Petri dishes (25 ml/dish) (Deploey and Fergus, 1975) was used to study the germinability of D. thermophilus conidia. An experiment was prepared by placing one drop of the spore suspension (20 drops = one ml) in a marked area of each Petri dish, then the drop was spread over about 15 square mm.

To study the effect of conidia concentration on germination six dilutions of washed conidia from D. thermophilus were prepared (50,000; 100,000; 150,000; 250,000; 500,000; 1,000,000 spores/ml). Petri dishes containing GB agar were inoculated as described above and incubated at 45C for 6 hr in a Model 17 Thelco Gravity Convection Oven (accurate to ± 0.5C). To determine percent spore germination for this and other studies a minimum of 300 randomly chosen spores were examined at 450X. A spore was designated as germinated if a germ tube formed that was as long as it was wide.

To determine the cardinal temperatures for spore germination six Petri dishes containing GB agar were inoculated with one drop of washed spore suspension. These six dishes were incubated at one of the selected temperatures (ranging from 18C to 60C) for varying times up to 72 hr. At the end of each incubation period one Petri dish was removed from the incubator and examined with a microscope (450X) to determine percent germination. If spores had not germinated the culture was incubated again at 45C to ascertain that the spores were viable. Cultures were also re-incubated in other experiments where no germination occurred.

The thermoduricity of D. thermophilus conidia was determined by placing one ml of the spore solution into each of three glass tubes (10 ml) pre-heated in water baths set at 55C, 60C and 65C (one tube per water bath). One drop of spore solution was removed from each of the three tubes at regular periods for up to 72 hr and placed on GB agar contained in Petri dishes. These dishes were incubated at 45C for 6 hr, and the percent spore germination was determined.

The ingredients of the liquid basal medium were also studied to

determine their effect on the germination of D. thermophilus conidia. The concentration of these nutrients when tested singly or in various combinations was the same as that given for the basal medium. Two other nitrogen sources (asparagine and $(NH_4)_2HPO_4$) were also studied singly at a concentration of 2 g/liter to determine their effect on the germination of these conidia. The nutrient solutions and spore suspensions were prepared at double the required concentrations allowing the two to dilute each other to the desired concentration when they were mixed in equal volumes. In addition, germination rates of these conidia was also determined on two undefined agar substrates (Difco corn meal and malt extract).

These experiments were repeated at least three times. All chemicals used were of reagent grade.

RESULTS

In this study (Table 1) the rate of germination by D. thermophilus conidia was unaffected by spore concentrations between 50,000 and 1,000,000 spores/ml. The germination rates were virtually identical for all spore concentrations ranging between 30.7% (250,000 spores/ml) and 38.7% (500,000 spores/ml).

Table 1. Effect of Spore Concentration on the Germination of Dactylomyces thermophilus Conidia.

Conidia Per ml of Suspension	Percent Germination[a]
50,000	34.0
100,000	33.7
150,000	37.0
250,000	30.7
500,000	38.7
1,000,000	32.9

[a]Incubation time was 6 hr at 45C.

The minimum temperature required for germination (Table 2) of D. thermophilus conidia was 20C, and germination was first observed in cultures incubated for 24 hr. Spores incubated at 18C for up to 72 hr did not germinate.

Table 2. Percent Germination of Dactylomyces thermophilus Conidia on Glucose-Basal Agar as a Function of Time and Temperature.

Temp. (C)	Incubation Time (hr)					
	4	8	12	24	48	72
18.0	0[s]	0[s]	0[s]	0[s]	0[s]	0[s]
20.0	0[s]	0[s]	0[s]	1.8	1.3	10.7
22.5	0[s]	0[s]	10.3	47.2	X[a]	X[a]
25.0	7.6	19.0	35.6	54.3	X[a]	X[a]
27.5	5.2	15.3	30.0	60.7	X[a]	X[a]
30.0	0[s]	14.4	12.8	38.6	X[b]	X[c]
32.5	12.5	30.9	57.5	X[a]	X[b]	X[c]
35.0	2.1	18.3	47.0	X[a]	X[b]	X[c]
37.5	10.0	12.0	48.7	X[a]	X[b]	X[c]
40.0	42.8	77.9	81.0	X[a]	X[b]	X[c]
42.5	44.5	76.7	74.8	X[b]	X[b]	X[c]
45.0	61.5	92.0	84.4	X[a]	X[b]	X[c]
47.5	22.3	52.9	80.1	X[a]	X[b]	X[c]
50.0	0[s]	0[s]	0[s]	1.9	X[a]	X[a]
52.5	0[s]	0[s]	0[s]	45.5	92.3	X[a]
55.0	0[n]	0[n]	0[n]	0[n]	0[n]	0[n]
57.5	0[n]	0[n]	0[n]	0[n]	0[n]	0[n]
60.0	0[n]	0[n]	0[n]	0[n]	0[n]	0[n]

[a]Germ tubes were too long and interwoven to determine percent germination, but ungerminated spores were seen (type a germination).
[b]Dense mycelial development; no spores seen (type b germination).
[c]Indicates germination was not determined.
[s]Spores survived when incubated again at 45C.
[n]Spores did not survive when incubated again at 45C.

The optimum temperature for germination (Table 2) of D. thermophilus conidia ranged between 42.5C and 45C. Although the germination rate was somewhat higher at 45C at the 4, 8, and 12 incubation periods, "a" and "b" type germination were recorded at the 24 and 48 hr incubation periods respectively.

The maximum temperature for germination (Table 2) of D. thermophilus conidia was 52.5C, but germination was not detected in cultures incubated for 4, 8, or 12 h. Furthermore, conidia incubated at 55C and above did not survive exposure to these conditions for 4 hr or longer.

Predictably, both thermoduricity and survivability decreased (Table

3) as the temperature and incubation time increased. Germination ceased when the conidia were incubated at 55C for 4 hr. In addition, no germination was observed when conidia were incubated for 15 min at 60C or for 5 min (0.0835 hr) at 65C, but usually some conidia were still viable since growth usually occurred in these cultures when they were incubated at 45C for an additional 24 hr.

Table 3. Thermoduricity and Survivability of Dactylomyces thermophilus Conidia Following Incubation in Distilled Water at 55, 60, and 65C for Up to 72 Hours.

Incubation Time (hr)	Incubation Temperatures (C)		
	55	60	65
0.0835	55.7	5.5	0^{ns}
0.25	33.6	0^{ns}	0^{ns}
0.5	17.9	0^{ns}	0^{ns}
1.0	6.6	0^{ns}	0^{ns}
2.0	1.8	0^{ns}	0^{ns}
3.0	1.3	0^{ns}	0^{ns}
4.0	0^{ns}	0^{ns}	0^{n}
5.0	0^{ns}	0^{ns}	0^{n}
6.0	0^{ns}	0^{ns}	0^{n}
7.0	0^{ns}	0^{ns}	0^{n}
8.0	0^{ns}	0^{ns}	0^{n}
12.0	0^{ns}	0^{ns}	0^{n}
24.0	0^{ns}	0^{ns}	0^{n}
48.0	0^{ns}	0^{ns}	0^{n}
72.0	0^{ns}	0^{ns}	0^{n}

^{n}Spores did not survive when incubated again at 45C on GB agar for 24 hr.
^{ns}Spores did not survive in some experiments but did survive in other experiments when they were incubated again at 45C on GB agar for 24 hr.

Conidia of D. thermophilus did not germinate (Table 4) in distilled or in aqueous solutions of the two inorganic nitrogen sources (KNO_3 and $(NH_4)_2HPO_4$) studied. However, they did germinate in aqueous asparagine as well as in solutions of glucose, biotin-thiamine, and KH_2PO_4-$MgSO_4$. Germination rates (Table 4) were virtually identical for conidia incubated on basal agar, corn meal agar, and malt extract agar.

Table 4. The Effect of Nutrients on Germination of Dactylomyces thermophilus Conidia in Liquid and on Agar Media (45C).

Medium	Agar Medium Percent Germination	Liquid Medium Percent Germination
Distilled water	-[b]	0[s]
KH_2PO_4-$MgSO_4$	-	17.8
Biotin-Thiamine	-	41.9
KNO_3	-	0[s]
$(NH_4)_2HPO_4$	-	0[s]
Asparagine	-	11.8
Glucose	-	53.5
Total Basal	93.7	-
Corn Meal	90.6	-
Malt Extract	92.4	-

[a]Incubation time was 6 hr.
[b]Dash indicates germination was not determined.
[s]Spores were viable when incubated again on GB agar at 45C for 24 hr.

DISCUSSION

Germination of D. thermophilus conidia involves swelling of the spores followed by germ tube formation. This process is affected by a variety of external influences, but the factors required by all fungal spores for germination include suitable temperature and pH, adequate moisture and oxygen, and viable spores (Lilly and Barnett, 1951). Cochrane (1958) also reported that spore germination can be influenced by spore concentration, but this effect was not observed in this study (Table 1) or in studies of the germination of spores from the thermophiles Rhizomucor pusillus (Deploey, 1989) and Chaetomium thermophile var. coprophile (Celerin and Fergus, 1971). Celerin and Fergus (1971) have suggested that the washing process used in preparing the spore solution may have removed substances in the cell wall that would have inhibited germination, but they did not investigate this possibility. Accordingly, studies are now underway in this laboratory to verify this hypothesis.

The cardinal temperatures for the germination of D. thermophilus conidia closely parallel the cardinal temperatures required for growth of thermophilic fungi as defined by Cooney and Emerson (1964). Temperatures required for the germination of spores from other thermophilic fungi have

also been reported and do not exceed the limits for growth. These include Chaetomium thermophile var. coprophile (Celerin and Fergus, 1971), Rhizomucor miehei (Deploey and Gautam, 1987), and R. pusillus (Deploey, 1989).

Conidia of D. thermophilus are less thermodurable than sporangiospores of R. pusillus (Deploey, 1989). Spores of R. pusillus were still germinable after a 12 hr exposure to 55C whereas conidia of D. thermophilus exposed to these conditions were still germinable after only 3 hr. Reduced germinability of D. thermophilus conidia (compared to R. pusillus) was also recorded when it was incubated at 60C and 65C. Conidia of D. thermophilus that did not germinate were incubated for an additional 24 hr at 45C to verify the viability of the spores, but conflicting results were obtained. In experiments done early in the study, spores that had not germinated were not viable when they were re-incubated at 45C for 24 hr. However, in identical experiments done later, spores subjected to the treatment for up to 72 hr (but which had not germinated) grew when they were re-incubated for 24 hr at 45C. The basis for this discrepancy is not clear and additional studies would be needed to account for these results.

Since the conidia of D. thermophilus did not germinate in distilled water, they would be designated as "nutritionally dependent" (Cochrane, 1958). The addition of certain inorganic (magnesium and phosphorus salts) and organic (glucose, vitamins, asparagine) nutrients induced germination, but solutions of either glucose or vitamins improved the germination rate most dramatically. Of the three nitrogen sources studied, germination occurred only in the asparagine solution. Neither KNO_3 nor $(NH_4)_2HPO_4$ induced germination. In the study done by Celerin and Fergus (1971) ascospores of C. thermophile var. coprophile germinated when incubated on agar containing either KNO_3 or $(NH_4)_2HPO_4$ (but no other nutrients). Unfortunately, they did not repeat this aspect of the experiment using liquid cultures making it difficult to compare their results (concerning utilization of nitrogen salts) with the present study. They did report, however, that C. thermophile var. coprophile was "partially nutrient dependent" since its ascospores germinated in distilled water whereas addition of nutrients improved the germination rate. In contrast Deploey (1989) reported that the sporangiospores of R. pusillus were "nutritionally dependent," but unlike D. thermophilus they germinated in only one of the substances (glucose) tested contained in liquid basal medium. Unfortunately, the present study of D. thermophilus conidia was not determined using total-basal liquid medium or water agar

made with distilled water and purified agar. A comparison of germination rates of D. thermophilus conidia incubated under these conditions with the data given in Table 4 would be useful in interpreting the results more precisely.

Celerin and Fergus (1971) and Doran (1922) reported that the age of a fungal culture can affect the viability of spores, and that this factor is species dependent. Ascospores of C. thermophile var. coprophile studied by Celerin and Fergus (1971) were more viable when obtained from older cultures. They assumed that the improved viability of the spores was linked to the completion of their maturation. In contrast, Doran (1922) reported that the viability of spores from the mesophilic fungi he studied decreased markedly as the spores age. The conidia of D. thermophilus studied here were obtained from four-day-old cultures and were at a stage of development permitting 100% germination when incubated properly.

Some investigators proposed that thermophily and spore thermoduricity are not necessarily correlated. Celerin and Fergus (1971) found that ascospores of C. thermophile var. coprophile survived 20 min at 65C. They also noted that spores of several mesophiles requiring heat activation for germination survived very high temperatures, but that heat resistance was lost once they were activated. However, one mesophilic ascomycete (Chaetomium cochliodes) not requiring heat activation to germinate survived a 2 min exposure to 80C (Dickson, 1932). Recently, Deploey (1989) reported that the sporangiospores of R. pusillus were less thermodurable than C. thermophile var. coprophile, being unable to germinate following a 5 min exposure to 65C. Some spores were still viable, however, having survived the 5 min and 15 min exposure to 65C when they were re-incubated (45C) for an additional 24 hr. As evidenced here, conidia of D. thermophilus were even less thermoduric than sporangiospores of R. pusillus. Although no extensive study of mesophilic thermoduricity is available, the studies cited here imply that there may not be a strong correlation between thermophily and spore thermoduricity. Additional studies of this subject would be needed to make a conclusive judgement.

SUMMARY

The effects of temperature, nutrients, and spore concentration on the germination of Dactylomyces thermophilus conidia were determined. A washed spore suspension was prepared from a tube slant culture. Spore concentration was adjusted to 500,000/ml (except in the spore

concentration study). Typically, one drop of this suspension was placed on glucose-basal (GB) agar contained in a Petri dish, the inoculated dish was incubated at 45C for 6 hr, and percent germination was determined. Six spore concentrations, ranging from 50,000 to 1,000,000 spores per ml, were studied to determine the effect of spore concentration on germination. Cardinal temperatures for germination were determined by calculating percent germination over a period of 72 hr at temperatures ranging from 18C to 60C. Spore thermoduricity was studied by incubating the spores in distilled water at temperatures of 55, 60, and 65C for as long as 72 hr and then determining the percent germination occurring after incubating these spores again at 45C for 6 hr on GB agar. The effects on spore germination of the components of the glucose-basal medium were determined by incubating spores for 6 hr in liquid cultures of various components of this medium.

Percent germination was not substantially influenced by the spore concentrations tested. The cardinal temperatures for germination are: minimum, 20C; optimum, 42.5-45C; maximum, 52.5C. Spores incubated in distilled water survived exposure to 55C for 3 hr, 60C for 5 min, but did not germinate after exposure to 65C for 5 min. Spores do not germinate in distilled water, in aqueous KNO_3, or in aqueous $(NH_4)_2HPO_4$, but do germinate in aqueous glucose, in aqueous biotin-thiamine, in aqueous KH_2PO_4-$MgSO_4$, and in aqueous asparagine.

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The Artificial Sweeteners, Aspartame and Saccharin, Alter *In Vitro* Bacterial Growth: A Preliminary Study

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INTRODUCTION

Several methods have been utilized by dental researchers to assess the effect that dietary components may have upon bacterial growth. Commonly enteric or ingested organisms are exposed to various concentrations or mixtures of biochemical substrates, and changes in growth, morphology, and reproduction are observed. The artificial sweetener aspartame has been shown not to alter metabolism of sucrose, glucose or fructose by Streptococcus mutans (Frank and Berry, 1984) in vitro. On the other hand, saccharin has been shown to decrease the growth of a mixed oral bacterial population in a dose-dependent manner (Grenby and Bull, 1979) while other studies have indicated that saccharin inhibits growth of gram positive and gram negative cocci without any effect on gram negative rods (Linke and Doyle, 1985). In this study, culture density patterns of specific bacteria resulting from exposure in vitro to either aspartame or saccharin are examined.

METHODS AND MATERIALS

Bacterial species used in this study were isolated from various animal sources. Pure cultures from nutrient agar plates were inoculated into thioglycollate media (Beeton, Dickinson and Co., Cockeysville, MD) to maintain a stock culture that was incubated at 20°C.

For the bacterial culture density experiments, inocula from the stock cultures were placed into separate 10% solutions of thioglycollate medium that contained 10 mg ml^{-1} aspartame or sodium saccharin. The volume of the inoculum from the stock solution was adjusted to provide an approximate starting culture density of 10^6 cells ml^{-1}. Cultures were grown in darkness at 20°C. Culture density was determined by changes in turbidity (660 nm) of a suspended culture on a Bausch and Lomb Model 21 Spectrophotometer and measured against a cell-free supernatant to account for removal of nutrients and release of cellular metabolites which also absorb at 660 nm. The spectrophotometer was calibrated with sterile culture media each day and culture sizes were determined. Within each treatment group, the culture size was expressed as a percent of initial density.

Data concerning culture density were collected on days 1, 3, 4 and 7. Inoculation of cultures was on day 1. This method was used to avoid the more rapid log-phase growth periods at higher temperatures which would obscure all but the most marked differences in culture size in the presence of saccharin or aspartame.

Data were considered statistically significant if the mean percent change in culture size was two or more standard deviations different from the control culture.

RESULTS

All bacteria isolated and cultured grew in a 1% thioglycollate medium to varying degrees (Table 1). Staphylococcus epidermis, Klebsiella pneumonia, and Escherichia coli increased to only 119.67%, 116.67%, and 123.67%, respectively of initial culture size. This is less than the other species tested.

Bacterial growth in a medium with 10 mg ml^{-1} aspartame caused an increase at day 3 of Erysipylothrix (insidiosa) rushiopathie, K. pneumoniae, and E. coli (Table 2). Furthermore, at day 5 bacterial culture size was increased by aspartame in Bacillus cereus, Streptococcus fecalis, and E. coli. On the other hand, at day 7, aspartame caused a decrease in B. cereus while Micrococcus spp. and E. coli were increased.

Bacterial growth in a medium with 10 mg ml^{-1} saccharin caused an increase in culture density at day 5 of B. cereus, Micrococcus spp. and E. coli (Table 3). By day 7 only Staphylococcus epidermis and Micrococcus spp. cultures were significantly increased.

Table 1. Bacterial Growth in a 1% Thioglycollate Medium[a].

BACTERIA	PERCENT CHANGE IN BACTERIAL CULTURE SIZE[b]			
	Day 1	Day 3	Day 5	Day 7
Bacillus cereus	100 ± 0.50	112.00 ± 7.93	110.60 ± 5.50	159.60 ± 8.50
Proteus mirabilis	100 ± 3.30	115.30 ± 8.08	120.00 ± 10.53	145.30 ± 0.57
Erysipylothrix	100 ± 0.70	106.67 ± 8.14	127.33 ± 12.05	139.00 ± 5.29
Streptococcus fecalis	100 ± 0.70	114.67 ± 8.32	115.33 ± 9.07	139.00 ± 12.16
Staphylococcus epidermis	100 ± 0.20	113.33 ± 7.24	118.60 ± 12.42	119.67 ± 17.47
Klebsiella pneumoniae	100 ± 0.50	101.00 ± 1.00	114.67 ± 4.93	116.67 ± 7.57
Micrococcus spp.	100 ± 0.20	110.67 ± 1.15	104.00 ± 2.00	130.00 ± 4.35
Escherchia coli	100 ± 0.20	104.67 ± 1.52	106.33 ± 3.78	123.67 ± 3.51

[a]A 1% thioglycollate medium was inoculated with approximately 10^6 cells ml^{-1} on day one.
[b]Bacterial culture size was determined as the percent of initial culture size on day one. Each point is presented as the mean ± S.D. (n = 3).

Table 2. Bacterial Growth in Media Containing 10 mg ml^{-1} Aspartame[a].

BACTERIA	PERCENT CHANGE IN BACTERIAL CULTURE SIZE[b]			
	Day 1	Day 3	Day 5	Day 7
Bacillus cereus	100 ± 0.50	110.67 ± 2.30	129.34 ± 1.52	138.34 ± 3.78
Proteus mirabilis	100 ± 3.30	114.00 ± 2.64	126.67 ± 4.16	142.00 ± 3.60
Erysipylothrix	100 ± 0.70	122.33 ± 1.52	129.34 ± 6.43	147.00 ± 3.46
Streptococcus fecalis	100 ± 0.70	123.67 ± 3.05	139.34 ± 4.51	139.00 ± 9.16
Staphylococcus epidermis	100 ± 0.20	109.30 ± 2.51	109.67 ± 3.78	120.67 ± 5.51
Klebsiella pneumoniae	100 ± 0.50	126.67 ± 3.05	119.67 ± 1.52	120.00 ± 0.81
Miocrococcus spp.	100 ± 0.20	121.00 ± 3.46	136.00 ± 2.00	144.67 ± 4.05
Escherchia coli	100 ± 0.20	118.67 ± 5.13	130.00 ± 3.10	140.00 ± 2.00

[a]Aspartame was purchased commercially and added to a 1% thioglycollate medium on day one. Each culture was then inoculated with approximately 10^6 cells ml^{-1}.

[b]Bacterial culture size was determined as the percent of initial culture size on day one. Each point is presented as the mean ± S.D. (n = 3).

Table 3. Bacterial Growth in Media Containing 10 mg ml^{-1} Saccharin[a].

BACTERIA	PERCENT CHANGE IN BACTERIAL CULTURE SIZE[b]			
	Day 1	Day 3	Day 5	Day 7
Bacillus cereus	100 ± 0.50	108.33 ± 1.15	133.00 ± 5.00	141.34 ± 14.70
Proteus mirabilis	100 ± 3.30	124.67 ± 1.15	135.34 ± 8.50	142.00 ± 6.00
Erysipylothrix	100 ± 0.70	116.30 ± 1.52	127.50 ± 2.08	135.00 ± 6.08
Streptococcus fecalis	100 ± 0.70	107.33 ± 2.08	122.33 ± 3.51	127.33 ± 2.51
Staphylococcus epidermis	100 ± 0.20	131.67 ± 2.88	138.00 ± 7.54	144.33 ± 6.65
Klebsiella pnuemoniae	100 ± 0.50	126.60 ± 9.86	140.33 ± 12.58	144.00 ± 7.55
Micrococcus spp.	100 ± 0.20	126.33 ± 3.05	139.00 ± 8.00	150.30 ± 9.23
Escherchia coli	100 ± 0.20	113.30 ± 4.61	119.30 ± 1.53	124.67 ± 2.31

[a]Saccharin was purchased commercially and added to a 1% Thioglycollate medium on day one. Each culture was the inoculated with approximately 10^6 cells ml^{-1}.

[b]Bacterial culture size was determined as the percent of initial culture size on day one. Each point is presented as the mean ± S.D. (n = 3).

DISCUSSION

The bacterial species used were chosen to provide a good cross section of biochemical and morphologic characteristics in animal pathogens and commensals. The artificial sweeteners do not appear to selectively alter any biochemical or morphological grouping of organisms. For example aspartame consistently increased the culture density of E. coli at all time points examined while P. mirabilis was unaffected. However in no case did the presence of the artificial sweetener inhibit bacterial culture density. It seems likely that the artificial sweeteners are serving as a metabolic substrate for the organism and the response as indicated by changes in culture density is a function of substrate utilization.

Saccharin and cyclamate have been shown to inhibit anaerobic fermentation of glucose by intestinal bacteria. Those authors have proposed that artificial sweeteners may act on the glucose transport system at the bacterial cytomembrane (Pfeffer et al., 1985). Other studies have shown that gram positive and gram negative cocci are inhibited by saccharin while gram negative rods are unaffected (Linke and Doyle, 1985). Furthermore, feeding rats saccharin, has been shown to have a bacteriostatic effect in the small intestine of the rat (Naim et al., 1985).

SUMMARY

A cross-section of representative commensal and pathogenic bacterial species were grown in culture with either aspartame or saccharin. The artificial sweeteners altered the growth of several species at various times after culture inoculation. There was no consistent pattern to the growth rate changes. However, it seems likely that both artificial sweeteners may serve as a substrate for the species examined.

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Protein Profiles of Mannitol-Shocked Soybean Cell Suspension Cultures

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INTRODUCTION

Water deficit stress causes numerous cellular and biochemical consequences in plants. When the individual consequences of water stress are expanded to a regional if not global level, they involve periods of drought and concomitantly reduced crop yields. Water deficit stress is induced by the addition of a nonpermeating osmoticum, such as either mannitol or polyethylene glycol (PEG) to the growth medium (Bressan et al., 1981; Bressan, et al. 1982; Handa et al., 1982; Hasegawa et al., 1984; Heyser and Nabors, 1981; Premecz et al., 1978).

Environmental stresses such as heat, drought, heavy metals, and salinity induce shock protein synthesis in plant tissues and cells (Heikkila et al., 1984; Kanabus et al, 1984; Key et al., 1985; Singh et al., 1985). Many size classes of proteins are induced by environmental stresses, but low molecular weight polypeptides of 15-20 kilodaltons (kD) are of interest because they are common to heat, drought, and salt stress in tobacco cell cultures (Harrington and Alm, 1988; Kanabus et al., 1984; Singh et al., 1985) as well as in other plants (Heikkila et al., 1984; Mansfield and Key, 1987). Polypeptides of 26 kD and 43 kD are common to tobacco cultures adapted to either PEG or NaCl (Singh et al., 1985). The 26 kD protein, now known as osmotin, has been purified from tobacco cells adapted to NaCl (Singh et al., 1987). Heat shocked soybean seedlings synthesize novel proteins in the 15-20 kD molecular weight range (Key et al., 1985;

Mansfield and Key, 1987). Hypertonic cells and protoplasts of Nicotiana sylvestris synthesize three proteins in the 30-50 kD Molecular weight range as well as one protein of less than 20 kD and one greater than 70 kD (Fleck et al., 1982). Pea root cells produce an enhanced quantity of a particular protein when grown in the absence of sucrose (Webster, 1980). Conversely, no novel shock proteins are found in PEG-stressed corn seedling mesocotyls, although heat shock mRNA synthesis occurs in response to the stress (Bewley et al, 1983; Heikkila et al., 1984).

Tomato cells, whether shocked or adapted to 15% PEG had a longer lag phase, gained fresh weight more slowly, and amounted to a smaller cell volume than untreated cells (Bressan et al., 1981; Bressan et al., 1982). Low levels of PEG (5% and 10%), stimulated dry weight gain in tobacco callus cultures (Bornman and Huber, 1979) and both dry weight and fresh weight gain in tomato suspension cultures (Bressan et al., 1981). Plant protein content is similarly affected by osmotic stress. Barley, stressed with mannitol (0.815 M) possessed reduced enzyme activities after six days of treatment (Coates and Davies, 1983). Both tobacco explants stressed with 10% PEG and protoplasts exposed to 0.4 and 0.7 M mannitol possessed decreased amounts of soluble protein in response to the osmotic stress (Bornman and Huber, 1979; Premecz et al., 1978). Mannitol causes a reduction in turgor in treated potato tuber disks (Oparka and Wright, 1988) and serves as an effective osmoticum for carrot and red beet cells (Cram, 1984).

Therefore, mannitol-shocked soybean cell suspension cultures were examined for shock-induced deterioration in the soluble protein profile and for the effects of osmotic shock on culture growth.

MATERIALS AND METHODS

Culture Conditions

Soybean cell suspension cultures (Glycine max, L. Merr. 'Wilkin'). originally obtained from the USDA-ARS Metabolism and Radiation Research Laboratory, Fargo, ND, were aseptically maintained on B5 medium (pH 5.6) (Gamborg et al., 1968) containing 1 mg 2,4-D l^{-1} to preserve friability. These cultures were kept in the dark at 25C with continuous gyrotory shaking at 100 rpm. Stock suspension cultures were subcultured weekly by pouring 5-10 ml of a mature culture (6-10 days old) into fresh medium.

Mannitol was added to the basal medium prior to pH adjustment to attain the final concentrations of 0.32 M and 0.64 M in the shock treatments. The treatment media were inoculated with 5 ml of cell

suspension culture in either the late log or early stationary phase of growth. All treatments were replicated three times.

Growth Measurements

The growth of the soybean cell suspension cultures was measured using settled cell volume, fresh weight, dry weight, and protein determinations. Settled cell volume was determined by pouring the entire culture into a graduate cylinder, rinsing the culture flask to remove the remaining cells, and allowing the cells to settle out by gravity. After 30 min, the volume of the cells was recorded (Davis et al., 1984). Cell suspension cultures were harvested after the prescribed stress time by vacuum filtration onto preweighed miracloth (Calbiochem, La Jolla, CA), washed for 15 sec with dH_2O to minimize any additional osmotic shock, and scraped onto tared aluminum foil to determine their fresh weights. Cells were washed for 1 min in dH_2O to remove all traces of the medium prior to drying for 24 hr at 70°C and weighed to determine culture dry matter production. Relative growth rates (RGR) were calculated according to:

$$RGR = \frac{[\ln(\text{final wt}) - \ln(\text{initial wt})]}{\text{time}}$$

in units of mg mg^{-1} d^{-1} (Glenn, 1987).

Protein Determination and Electrophoresis

Suspension cultures were harvested as previously described, resuspended in 10 ml of 0.05 M KH_2PO_4 (pH 7.5), and homogenized with a Ten Broeck tissue homogenizer. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 8000 x g in a Beckman JA-20 rotor for 10 min to sediment cell debris. The supernatant was treated with an equal volume of cold 10% trichloroacetic acid, centrifuged at 10,000 x g for 10 min, and the pellet solubilized in 0.1 N NaOH. All of the preceding steps were carried out at 0-2C. Lowry et al. (1951) protein assays were performed on 0.2 ml triplicate samples with BSA as the standard. Protein was quantified as mg per culture.

Water-soluble proteins for electrophoresis were extracted from cell cultures in 2 ml of 0.05 M Tris-HCl (pH 8.3) containing 1% (v/v) β-mercaptoethanol, 2% (v/v) sodium dodecyl sulfate (SDS), and 1 mM phenylmethysulfonyl fluoride, centrifuged at 10,000 rpm for 30 min, and filtered through miracloth (Calbiochem; La Jolla, CA) (Erickson and Alfinito, 1984; Lin, et al, 1984). Protein was assayed according to the Bradford (1976) method after precipitation and solubilization

of the protein as above. An aliquot of the protein sample was treated with an equal volume of 0.125 M Tris-HCl (pH 6.8) 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.05% bromphenol blue and heated to 100C. Sixty μg of protein was loaded per lane. The polypeptides were separated in a modified Laemmli gel system (1970) consisting of a 4% (w/v) acrylamide stacking gel and 13.5% (w/v) resolving gel. The gels were run for 1 hr at 35 mAmp prior to sample analysis and run for 2-3 hr during sample analysis (Erickson and Alfinito, 1984). All gels were stained in 0.4% (w/v) Coomassie blue in 14% acetic acid, 20% methanol (v/v), and 73% (v/v) water. Quantitative protein changes were determined by densitometric scanning at 595 nm of the dry gels with an LKB 2202 laser densitometer.

RESULTS AND DISCUSSION

Culture Growth Under Stress

Relative growth rates of mannitol-stressed soybean cell suspension cultures indicate that the mild osmotic shock of 0.32 M mannitol caused a slight reduction in growth of 4% for settled cell volume and of 10% for fresh weight during the duration of the experiments (Table 1).

Table 1. Relative Growth Rates of Mannitol-Shocked Soybean Cell Suspension Cultures.

	Mannitol Concentration (M)		
Parameter	0.00	0.32	0.64
Settled Cell Volume[a]	0.27[b]	0.26	0.11
Fresh Weight	0.32	0.29	0.08
Dry Weight	0.24	0.27	0.11
Protein	0.18	0.20	0.06

[a]Data were calculated from settled cell volume (SCV, ml ml^{-1} d^{-1}), Fresh weight (FW, mg mg^{-1} d^{-1}) dry weight (DW, mg mg^{-1} d^{-}), and protein (P, mg mg^{-1} d^{-1}).

[b]Values are means where n=3.

This reduction in growth of the mildly shocked cultures is attributed to water loss which reduced both cell volume and cell mass. Growth rates based on dry weight and protein accumulation are stimulated by 12% and 11%, respectively in the presence of the low concentration of mannitol (Table 1). Stimulation of growth under relatively mild stress conditions is not uncommon (Bornman and Huber, 1979; Bressan et al., 1981) and indicates that acclimation to water deficit stress occurs. Growth rates of the severely shocked cultures (0.64 M mannitol) were considerably reduced and the cultures did not acclimate to the mannitol treatment.

Control culture growth peaked at five days whether determined by settled cell volume, fresh weight, dry weight, or protein accumulation (Figures 1, 2, and data not presented); while the growth of the mildly shocked cultures was still increasing. Although, not significantly different from the controls for either fresh weight or protein, the growth of the mildly stresed cultures surpassed that of the controls at seven days. The overall pattern of growth is similar with regard to the growth parameter measured. Significant differences between the treatments were not evident until three days. The mildly shocked cultures have extended lag and log phases of growth. They remain in log phase at seven days, but they approach (Figure 1) or begin to exceed (Figure 2) the growth of the control cultures now in stationary phase. Culture growth peaks at five days in the severely stressed cultures; however, cultures do not recover from the osmotic shock (Figures 1 and 2).

Since 0.32 M mannitol stimulates soybean cell culture growth and 0.64 M mannitol inhibits culture growth, a threshold concentration at which cells can recover from the shock and grow similarly to the control cultures must be between these two values. Tobacco protoplasts at an intermediate concentration of 0.4 M mannitol possess reduced protein contents (Premecz et al., 1978), which indicates that the threshold mannitol concentration for soybean cells might be in this range.

Electrophoretic Analysis

Since no significant differences in cell culture soluble protein content are detected after one day and that the mildly stressed cultures (0.32 M mannitol) acclimate to the stress within five days (Figure 2), protein was extracted from one and three day old experimental cultures and analyzed via electrophoresis for shock protein production (Figure 3). A 17.0 kD protein appears in the treated cultures in an enhanced quantity (Figure 2). A 15.5 kD

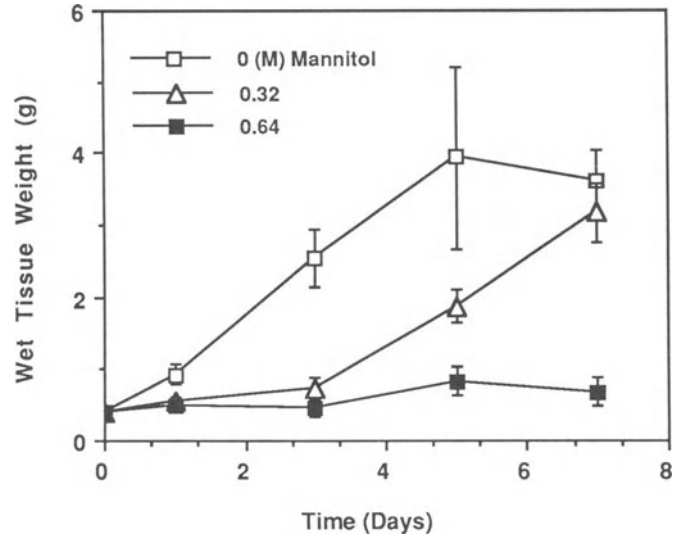

Figure 1. Growth of Mannitol-Shocked Soybean Cell Suspension Cultures as Measured by Culture Fresh Weight. Data are means (±SD), n = 3.

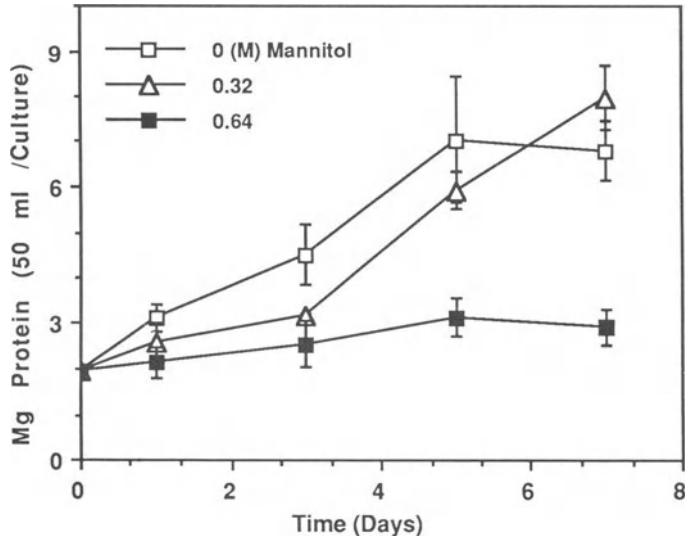

Figure 2. Growth of Mannitol-Shocked Soybean Cell Suspension Cultures as Measured by Soluble Protein Content. Data are means (± SD), n = 3.

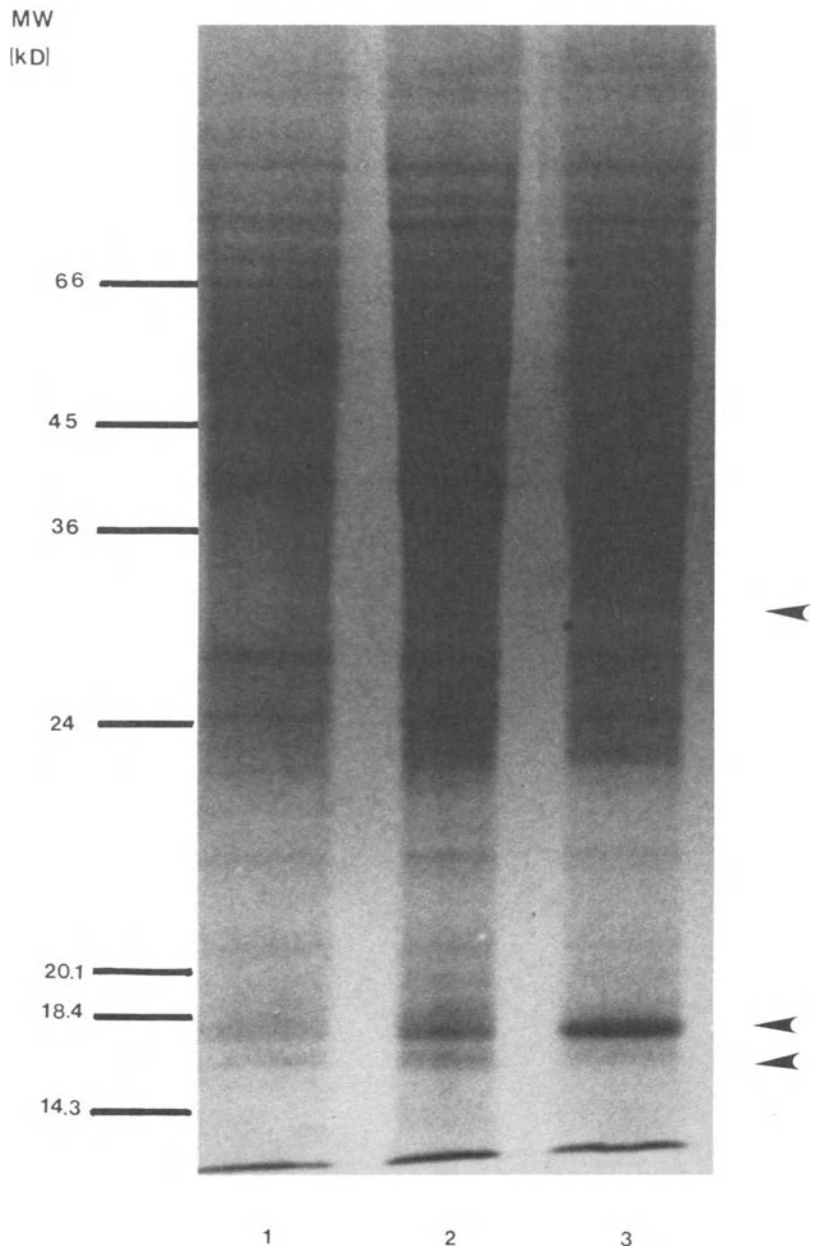

Figure 3. Protein Banding Patterns of Polypeptides from Three-day Old Cultures. Control cultures (Lane 1), 0.32M mannitol treated cultures (Lane 2), and 0.64 M mannitol treated cultures (Lane 3). Arrows indicate bands of 34.0 kD, 17.0 kD, and 15.5 kD.

protein is also synthesized in an enhanced quantity in these cultures. Both of these proteins are present at low levels, which cannot be quantitated with laser densitometry, in the control cultures after one and three days of growth. These protein bands, although visible to the unaided eye, do not necessarily resolve into distinct bands with laser densitometry. In the mild stress treatment the 15.5 kD band amounted to 0.1% of the protein applied to the gel and the 17.0 kD band consisted of 0.2% of the detectable protein. No novel shock proteins were detected in these cultures; however, a 34.0 kD. band can be seen only in the mannitol-treated cultures (Figure 3). This band, although not visible in the control culture lane, was detectable with densitometric analysis and amounted to 1.1% of the total protein. In the severely stressed cultures the 15.5 and 17.0 kD bands collectively made up 3.1% of the applied protein since they did not resolve into two distinct bands. The 34.0 kD band, although visible in the severely stressed treatment, was also not quantitated with laser densitometry.

Low molecular weight proteins in the 15-20 kD range are associated with heat shock (Cooper et al., 1984; Key et al., 1985; Mansfield and Key, 1987). Proteins of 18 and 19.5 kD are found in tobacco cells adapted to NaCl and bands of 17.5 and 16.5 are detected in PEG adapted tobacco cultures (Singh et al., 1985). The function of these low molecular weight proteins is unknown, but heat shock proteins confer thermotolerance in high temperature treated tissues and may perform a regulatory function (Key et al., 1985; Lin et al., 1984; Mansfield and Key, 1987). The protein bands associated with mannitol-induced osmotic shock have similar sizes to those associated with heat shock and adaptation to NaCl or PEG. Since the cultures treated with 0.32 M mannitol are capable of acclimating to the stress, these proteins may bestow stress tolerance in cell cultures of soybean and because of their small size they may be part of a universal response to environmental stress and deterioration.

SUMMARY

Soybean cell suspension cultures shocked with 0.32 and 0.64 M mannitol produce enhanced levels of 15.5 and 17.0 kD polypeptides after 1 and 3 days of stress treatment. No unique proteins arising from de novo synthesis were detected. Although, a polypeptide of 34.0 kD did resolve in the shock treatment extracts, it remained unresolved in the control. The mild mannitol shock of 0.32M stimulated culture relative growth rates by 11 and 12% for culture

total soluble protein and dry weight, respectively, indicating that these cells are capable of acclimating to the 0.32 M mannitol treatment within seven days. Culture relative growth rates for settled cell volume and fresh weight were reduced by only 4% and 10%, respectively. Water loss in these cultures is likewise not severe since cultures begin to recover fresh weight and cell volume by seven days. Relative growth rates of the severely shocked soybean cell suspensions (0.64 M mannitol) were reduced from 60%-80% when compared with the untreated cultures. Cultures do not recover from the severe shock of 0.64 M mannitol and may in fact be dead. Generally, the deteriorative effects of the mannitol shock on culture growth were not evident until the third day of stress treatment.

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Serotonin Toxicity Causes Myodeterioration of Skeletal Muscle Function in Albino Mice

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INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) functions as a neurotransmitter affecting a variety of tissues, including gastrointestinal smooth muscle and striated limb muscle (Bulbring and Gershon, 1967; Ooms et al., 1986). It has also been shown to be a potent vasoconstricting agent (Carter, 1961). As a vasoconstrictor, serotonin causes decreased blood flow through capillaries to skeletal muscles. The decreased oxygen and nutrient exchange between blood and the "isolated" skeletal musculature results in changes in both histological appearance and muscle function (Erspamer, 1961) . However, evidence suggesting that serotonin acts more directly on muscle cells has been reported. Attention has been given to studies testing the involvement of receptors responding to 5-HT stimulation in both vascular smooth muscle and skeletal muscle in an effort to discern the receptor subtype which may be in part responsible for the serotonin induced muscle myopathy. Interest has developed in attempting to determine which antagonists for distinct serotonin receptors will be effective therapeutically when administered to animal subjects suffering from serotonin induced myopathies.

It has been reported from studies using rats (Verheyen et al., 1981) and horses (Ooms et al., 1986) that oral preparations of ketanserin and ritanserin (S_2 serotonin receptor antagonists) significantly reverse skeletal muscle myopathies induced by daily intraperitoneal (I.P.) injections of serotonin. The studies reported treatment periods ranging from 5 to 60 days which provided information on both chronic and acute

responses. In the acute rat studies, the femoral artery was unilaterally ligated prior to drug treatment, while the chronic studies involved the treatment of horses with pre-existing myopathies without arterial ligation.

Considering the results from the acute and chronic studies in which serotonin was shown to induce muscle deterioration and the receptor antagonists were reported to promote healing of the myopathies, it would appear that multiple S_2 receptors are involved. If serotonin were to act on vascular smooth muscle alone, reducing blood flow to the area in question, it is logical that antagonist treatment on animals with ligated femoral arteries would not affect the progression of the myopathy. The ligated rats, according to Verheyen, showed marked improvement with 5-HT antagonist therapy even with femoral artery ligation. Since the acute phase using rodents required arterial ligations and relatively low concentrations of the therapeutic drugs (the antagonists), it became apparent that another study would be needed to determine whether S_2 antagonist-reversals of the serotonin-induced lesions would occur acutely in non-ligated animals. It was also conceivable that the newer, more specific S_2 antagonist, ritanserin might prove more effective than ketanserin in these tests.

MATERIALS AND METHODS

Young adult albino mice of both sexes were used in these experiments. In addition to the control animals, receiving drug vehicle only (VO), the mice were divided into three groups for study, serotonin-treated (ST), serotonin and ketanserin-treated (SKT), serotonin and ritanserin-treated (SRT), ketanserin only (KO), and ritanserin only (RO). All mice were maintained at ambient room temperature (23-25^{o}C) and fed standard laboratory rodent chow. Antagonist drugs were added to the appropriate rodent chow containers. These drugs were mixed into ground laboratory chow so that each animal received consistent quantities of drug daily (5-10 mg/kg b.w.). The experimental mice (with the exception of the KO and RO treatment groups) were injected daily with IP doses of 20 mg/kg b.w. of serotonin. After five days of treatment, the mice were lightly anesthetized and then sacrificed by cervical dislocation. The muscle preparations consisted of the entire left hindlimb of each animal. The muscle preparations were connected to a myograph transducer in-line with a Harvard Chart Mover Recording System. The calibration was adjusted on each muscle preparation until a standard calibration of 10

mm/g of contractile force was obtained for each preparation. These calibrations were set so that a given force applied to the muscle transducer would cause a corresponding vertical deflection by the recorder pen. Normal mammalian body temperature ($37^{o}C$) was maintained throughout the experiment by perfusing the muscle preparations with oxygenated mammalian Tyrode's Solution.

Each muscle preparation was stimulated successively with a series of electrical shocks applied at a frequency of 1/0.5 sec, 5V and durations of 15 msec. The stimulus was applied for several 10-second intervals until maximum muscle treppe occurred. Contracture plateaus were then determined by observing the recovery from fatigue by individual peaks. The plateau (peak height) values were measured in millimeters (mm). Each treatment group was analyzed for outliers by the Q-test and those values found to be unreliable were eliminated. Each data group was compared to one another by computer aided analysis of variance. In all analyses, the serotonin treated animals showed significant differences from the other treatment groups. For easier presentation, the data groups were expressed in the text and tables as a percentage of the control value. The control group was assumed to be 100%.

RESULTS

A comparison of percentage difference from the non-treated controls with each of the drug treated groups has been given in Table 1. It was clear from this illustration that a significant decrease in skeletal muscle contractility occurred in all animals that received serotonin only, and that significant increases occurred in all groups receiving either serotonin and antagonists or antagonists only. It has been shown by these data that both antagonists, functioning independently (not involving pre-treatment with serotonin), may increase normal muscle activity by nearly 50%.

Upon comparing the contraction data from male animals with that of females (Table 2), it was apparent that the latter respond more forcefully to stimulation by the antagonist drugs following pre-treatment with serotonin. The mean responses due to ketanserin and ritanserin only were compared. It was shown that a proportional increase in the male contraction value over that of the control value occurred and was considerably greater than were values recorded for the female. In summary, it appears that the female mice tend to achieve a higher

Table 1. Percent of Control Contraction Value for Drug Treated Animals.

Treatment Group	Percent of Control[a]
Serotonin Treated (ST)	41 %
ST + Ketanserin	166 %
ST + Ritanserin	138 %
Ketanserin Treated Only	138 %
Ritanserin Treated Only	132 %

[a]This represents a comparison of individual treatment groups to the control group. The percentages expressed are percentages of the mean value obtained for the control treatment group.

Table 2. Percent of Control Contraction Value for Drug Treated Animals Separated by Sex.

Treatment Group	Percent of Control	
	Male	Female
Serotonin Treated (ST)	39 %	41 %
ST + Ketanserin	95 %	159 %
ST + Ritanserin	99 %	158 %
Ketanserin Treated Only	145 %	109 %
Ritanserin Treated Only	132 %	125 %

contractile value than corresponding male animals following the administration of the serotonin antagonists and serotonin. The males appear to be more susceptible to serotonin alone, yet show a greater percentage increase in skeletal muscle contraction than female mice when treated with ketanserin or ritanserin, only.

DISCUSSION

The results from this study have shown that serotonin when given IP at higher than physiological concentrations, induces myopathy development in the skeletal musculature of albino mice. The acute toxicity of serotonin may be promptly reversed by treatment with specific serotonin S_2 receptor antagonists. There have been no previous reports in the literature of such a diversity of changes as were observed in the current study in connection with the observed effects of antagonist drugs on limb muscle motility tested In vitro. Although other investigators (Verheyen et al., 1981; Ooms et al., 1986) have shown that when certain serotonin antagonists were fed orally to rats during both acute and chronic investigations, special conditions had to be met in order to achieve a significant degree of myodeterioration. One of the major conditions of the previous study by Ooms, et al. (1986) was the ligation of the femoral artery prior to drug treatment. As no vascular ligation was done in the current study, we were able to postulate from our data that different serotonin receptors may mediate varying responses in mammalian skeletal muscle. These responses were quantified via a novel approach employing perfusion of whole limb muscle preparations attached to a physiological recording system (Kimbrough and Llewellyn, 1988).

In view of the recent reports by Branchek (1988), Campbell (1989) and Clarke (1989), we find that a great deal of data now supports the premise that multiple binding sites exist for serotonin in peripheral organs e.g. intestinal viscera and skeletal muscle. One of the recently described serotonin receptors, found in the lamina propria of the mammalian gut mucosa, is $5\text{-}HT_{1p}$ which has a high affinity for $^3H\text{-}5\text{-}HT$, initiates longer-lasting depolarization of enteric neurons, and is the physiological receptor through which serotonergic neurons in the intestinal tract mediate slow excitatory post-synaptic potentials (EPSPs) (Branchek, Mawe, and Gershon, 1988). Branchek, et al. had previously reported that the $5\text{-}HT_3$ ($5\text{-}HT_{2p}$) receptors bind serotonin with low affinity and initiate a brief depolarization of enteric neurons with decreased input resistance. No physiological action of serotonin was

reported to be mediated by these receptors at that time. However, other investigators later reported that a class of peripheral receptors, known as sigma receptors were highly localized and specific binding proteins in mammalian brain and that these were 5-HT3 receptors (Campbell, et al., 1989). Still later, it was reported that a number of novel sigma receptor-active drugs derived from guanidine (known as DTGs), were shown to inhibit contractions of the guinea pig ileal longitudinal muscle/myenteric plexus preparation (LMMP) evoked by electrical stimulation. It was also found that this class of DTG drugs noncompetitive antagonized contractions of the LMMP preparation caused by serotonin. Of further interest has been the very recent discovery of yet another peripheral 5-HT receptor, called 5-HT_4 (Clarke et al., 1989). This binding protein's positive link to adenyl cyclase was first studied in the guinea pig limbic system, but attention is now being called to its location in the ileum where antagonist potency has been shown to be significantly greater. The rationale for including the 5-HT_4 receptor in this discussion stems, however, from the yet unpublished findings by Kaumann, et al., which suggest such receptors may also exist in the cardiovascular system (Kaumann, 1990). With this new location in mind, it could be speculated that the 5-HT_4 receptors might represent one of the binding proteins that respond to serotonin's stimulation in the arterial wall of the blood vessels that supply skeletal muscle.

From the data obtained in this study, it would appear that from an overall standpoint, ketanserin is the most effective antagonist, considering its rapid action in initiating the recovery from serotonin-induced myodeterioration of skeletal muscle. Yet, ritanserin (also used in this study) like ketanserin, is an S_2 receptor antagonist, and from recent reports, it has a greater affinity and specificity for serotonin binding sites than ketanserin. This apparent incongruity remains unresolved. However, as based on the composite of the findings from this study, some different experimental approaches have been identified which may reveal new insight into the treatment of serotonin-induced myopathies in skeletal muscle.

SUMMARY

Although it has been assumed by some investigators that skeletal muscle myopathies are induced by the toxic action of serotonin (5-hydroxytryptamine) acting alone at vascular smooth muscle receptors, recent studies have suggested the possibility of multiple binding sites. Serotonin, when given intraperitoneally at a dosage of 6 mg/kg body

weight per day, for five days, not only retards hind limb motion, but causes a state of general flaccidity and debilitation of the skeletal musculature. Albino mice were treated with one of two antagonists which were specific for the 5-HT_2 receptor. Ketanserin and ritanserin were given ad libitum during a three-phase study. Each phase lasted five days, during which time the drugs were mixed into rodent chow so that the animals ingested quantities of the antagonists confirmed to be within the range of 5-10 mg/kg b.w. per day for five days. Not only did the ketanserin and ritanserin treatments promote reversal of the myopathies, but in some instances, they increased the force of the limb muscle contraction. Post-treatment values for limb muscle contractility were recorded as high as 66% above control for ketanserin and 38% above control for ritanserin.

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Antihelminthic Activity of the Drug, Oltipraz, on the Surface Ultrastructure of *Schistosoma mansoni*

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INTRODUCTION

When schistosomes and cestodes are exposed to unfavorable environmental conditions, one of the most important anatomical components that undergoes morphological and degenerative changes is the outer body wall (surface tegument). The primary function of the surface tegument is to mediate the intake of nutrients from the host environment. In addition, it facilitates the secretion of enzymes and regulates the ionic flow of water (Smyth, 1969; Lumsden and Specian, 1980; Threadgold, 1984; Jones et al., 1989; 1988, 1977a and 1977b). Concomitantly, the surface tegument provides a shield for the host and an immunological mechanism, thus allowing resistance to foreign substances (Lumsden et al., 1980). The structure of the surface tegument of schistosomes, particularly Schistosoma mansoni, has been studied utilizing ultrathin sections for transmission electron microscopy (TEM) (Senft et al., 1961; Silk et al., 1969; Smith et al., 1969; Lumsden and Specian, 1980) and by scanning electron microscopy (SEM) (Miller et al., 1972; Hockley, 1968; Johnson and Moriearty, 1969; Robson and Erasmus, 1970). These investigations have revealed that the surface tegument is a syncytium made up of a surface layer recognized as a cytoplasmic sheath or matrix, connected by cellular extensions to cell bodies (nuclei) buried in the parenchyma.

Although a limited number of investigators is studying the molecular basis of the activity of antihelminthic drugs, it is becoming more obvious that mode and site of action studies are essential for providing a better understanding of the physiological and the biochemical systems in these

parasites. Two of the most recently used antihelminthic drugs that cause degradation and deterioration of the surface tegument of schistosomes that eventually lead to death of the parasite are praziquantel and oltipraz (Thomas et al., 1978; Horchner 1979; Anderson et al., 1985; Thomas et al., 1982; Shaw et al., 1983; Kardoman et al., 1985; Jones et al., 1988; 1989; Lapierre et al., 1983; Massoud et al., 1984a and 1984b). Previous studies have shown oltipraz to be a slower acting compound in comparison to praziquantel (Cerf et al., 1982; Bella et al., 1982; Lapierre et al., 1983; Xiao et al., 1984; Bieder et al., 1985). However, the administering of oltipraz to infected individuals causes a substantial reduction in schistosomes eggs. The mechanism of action of oltipraz is not clear but it has been suggested that it might inhibit the parasite's glutathione levels (Cerf et al., 1982; Bella et al., 1982; Lapierre et al., 1983; Rey et al., 1984; Kardoman et al., 1985; Bieder and Jumeau, 1985). The present study was undertaken to investigate the *in vitro* effects of oltipraz on the surface tegument of *S. mansoni* using scanning electron microscopy. Subsequently, X-ray Energy Spectrometry (XES) was used for elemental analysis.

MATERIALS AND METHODS

Animal Care

Animals were housed and maintained at the Center for Laboratory Animal Resources located at the Science Research Institute of the Atlanta University Center. The animals were kept in a constant temperature room with a temperature of $25^{o}C \pm 20^{o}C$, and housed in metal cages with wood shavings and exposed to breeder chow and water at all times. Eight week old *S. mansoni* pairs were isolated from the portal vein of both male and female mice (obtained from the University of Lowell; Lowell, MA) that had been anesthetized with sodium pentobarbitol to cause a complete shift of worms from the mesenteric vessels to the portal vein branches in the liver. After isolation, the worms were divided into several groups (5 to 10 per group), incubated for various times (30, 60, 90 and 150 min) at $37^{o}C$ with gentle agitation in solutions containing various concentrations of the anti-schistosomia drug, oltipraz (25, 50, 100, 150, 250, 500, 1000 and 1500 µg/ml. Control worms were incubated parallel to experimental worms in equivalent amount of perfusion fluid.

Electron Microscopy

Following incubation, *S. mansoni* worms were prepared for scanning electron microscopy. The worms were fixed for 6 hrs in 3% glutaraldehyde buffered with 0.1 M sodium cacodylate at pH 7.4, post fixed in buffered 2% osmium tetroxide and dehydrated in a graded series of ethyl alcohol. The

specimens were then critically point dried in carbon dioxide. The dried specimens were mounted on metal stubs, sputter-coated with gold/palladium and observed with an ETEC-Omniscan scanning electron microscope. Pictures were taken at desirable magnifications.

RESULTS AND DISCUSSION

Male and female Schistosoma mansoni worms treated, in vitro with various concentrations of oltipraz at various time intervals showed pronounced alterations, or changes on the surface tegument as compared to the normal worms. These alterations became more evident as the concentration and incubation time of the drug was increased. A photoelectron micrograph of an intact male and female S. mansoni worm is depicted in Figure 1. The anterior and posterior end of the female worm is attached in the canalis gynecophorus of the male worm. The surface tegument of the female worm consists of a relatively smooth surface tegument that is devoid of spines and bosses. Surface topography of the male worm includes the presence of oral and ventral suckers, canalis gynecophorus and bosses with and devoid of spines (Figures 1, 2 and 3).

Male and female worms treated with 25 µg/ml of oltipraz at intervals ranging from 30 to 60 minutes exhibited little tegumental damage (Figure 4). However, worms treated with 25 µg/ml of oltipraz for 12 hours showed pronounced damage to the surface tegument (Figure 5). Other tegumental surface alterations observed included progressive shrinkage of the oral suckers (Figure 5A) and deep grooves and depression on the dorsal side of the worm (Figure 5B). Subsequently, the anterior end of the worms treated with high dosages of the drug (1000 µg/ml and 1500 µg/ml for 60 minutes and 150 minutes) showed shrinkage and degeneration of the tegument (Figure 6). At high magnifications, the lower neck region showed irregularities in size, shape and distribution of spines and bosses (Figure 7). In some areas, at concentrations of 1000 µg/ml and 1500 µg/ml for 60 minutes and 150 minutes, necrosis of the tegument was apparent (Figures 8A-B).

The mid-body region exposed to 25 µg/ml and 50 µg/ml of the drug for long periods of time showed bosses that appeared more pointed and erect and folding of the tegument was evident (Figures 9A-B). However, bosses in other areas of the tegument were more widely distributed on the surface. Incorporation of 1000 µg/ml and 1500 µg/ml of oltipraz revealed a ruffled surface tegument and the bosses showed the appearance of moving inwards (Figures 10A-B).

Spines located on the posterior end of the parasite appeared irregularly distributed on the surface after exposure to 25 µg/ml and 50 µg/ml of the drug (Figures 11A-B). Worms treated with 1000 µg/ml and 1500

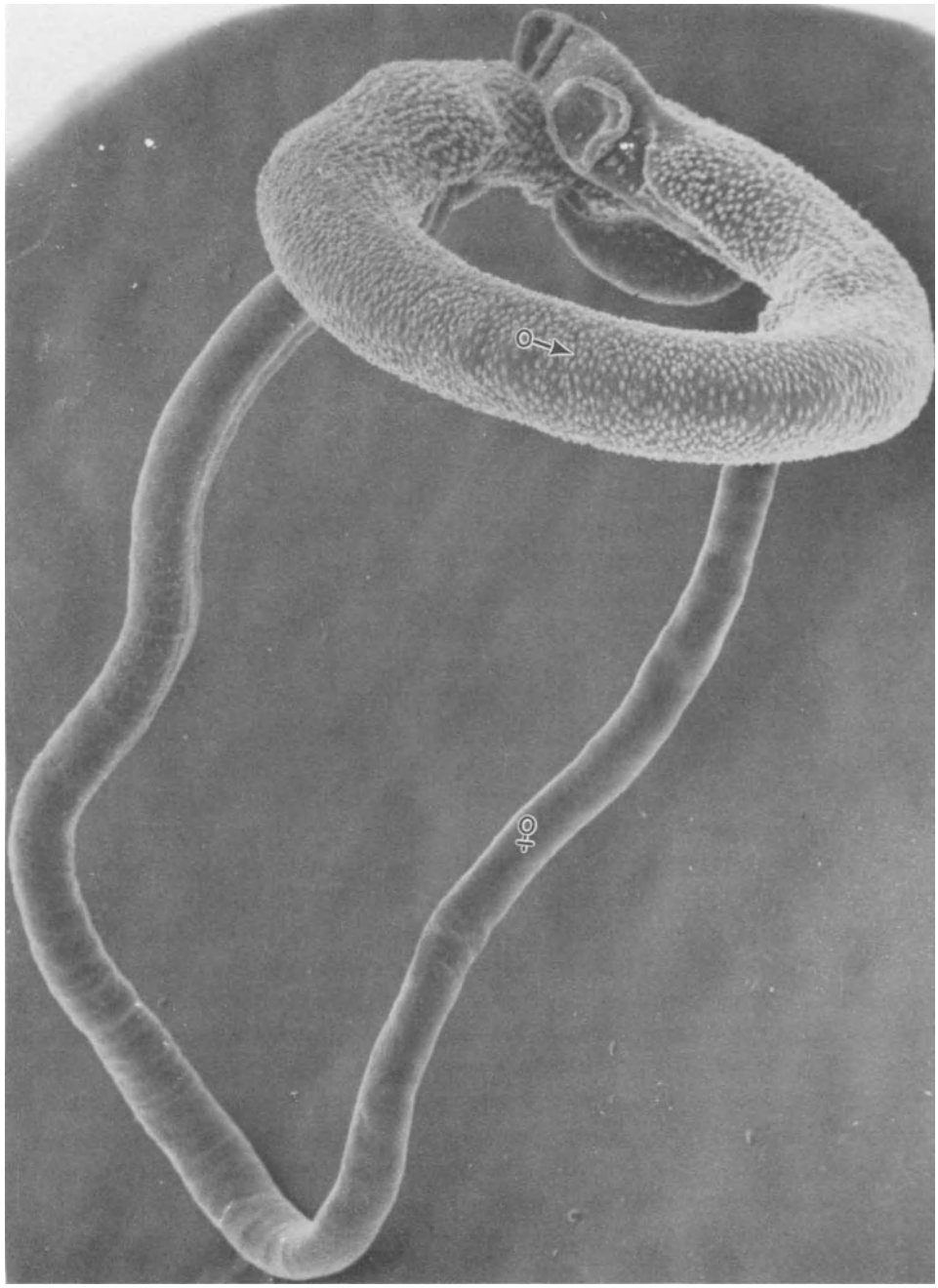

Figure 1 Scanning Electron Micrograph Scan of an Intact Male (♂) and Female (♀) Schistosoma mansoni. Note: Both the Anterior End and the Posterior End of the Female Worm Are Attached to the Male Canalis Gynecophorus. X70.

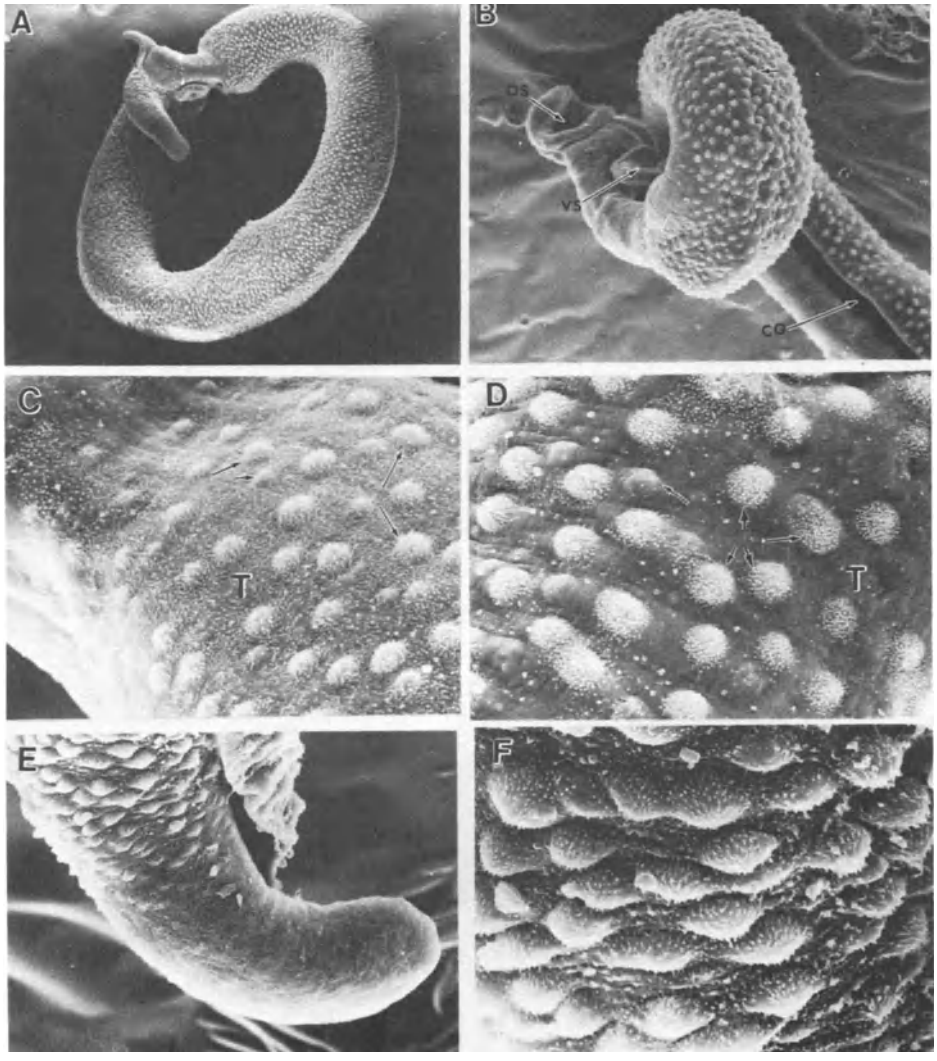

Figure 2 Scanning Electron Micrograph of a Normal Schistosoma mansoni Worm. (A) Intact Schistosoma mansoni Male Worm X46, (B) The Anterior End Revealing the Oral Sucker (OS) Ventral Sucker (VS) and Canalis Gynecophorus (CG) X142, (C) Lower Neck Region of the Worm Depicting the Tegument (T) with Spine and Spineless Bosses (arrows) X575, (D) The Mid-body Region that Consists of Spine and Spineless Bosses (arrows) X493, (E) The Posterior End (Tail Region) Consist of Spines on the Tegument X223, and (F) Micrograph Showing the Area Located Immediately Above the Tail Revealing Bosses with Spines. X446.

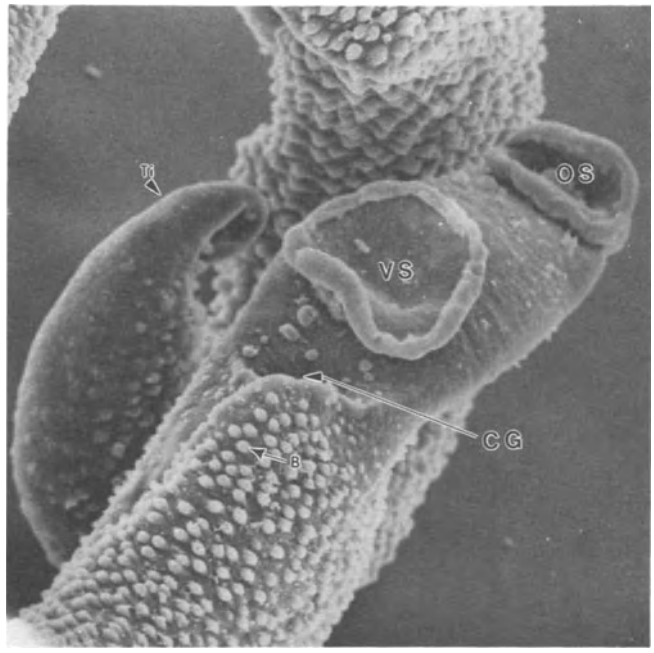

Figure 3 Scanning Electron Micrograph of the Male Anterior Section that Consists of the Oral Suckers (OS), Ventral Suckers (VS), Canalis Gynecophorus (CG) and Bosses (B). The Posterior Section Depicting the Tail Region (Ti) Can Also Be Seen. X342.

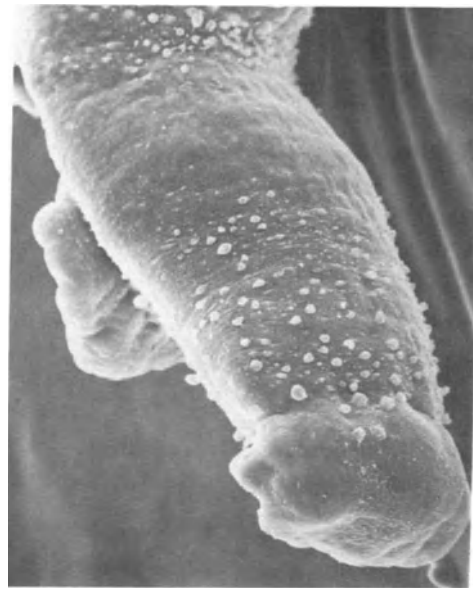

Figure 4 Scanning Electron Micrograph showing the Anterior End Schistosoma mansoni treated with 25 µg/ml for 12 hrs. X272.

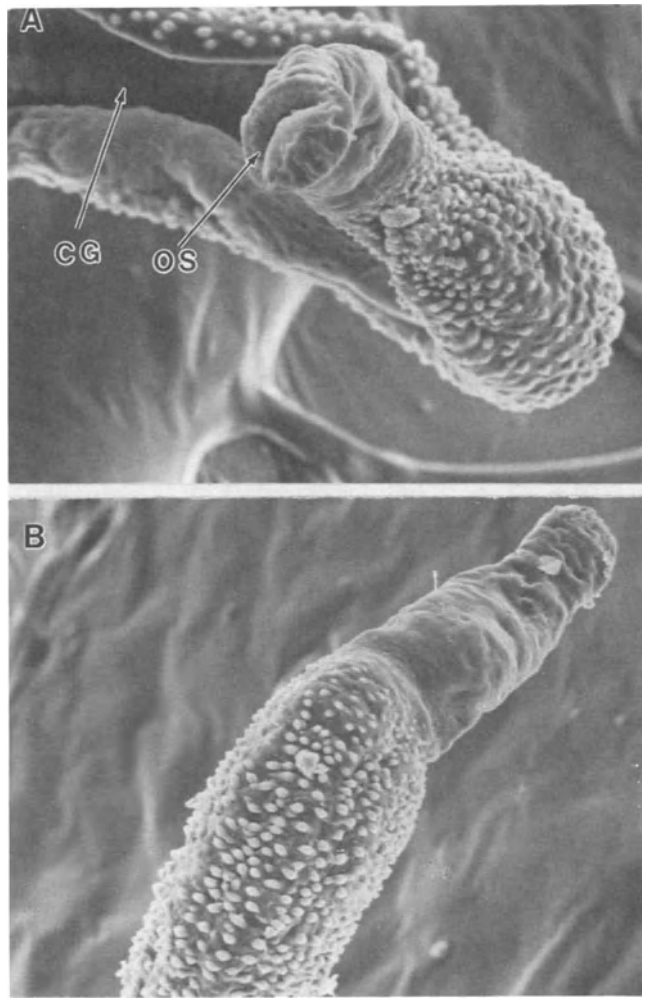

Figure 5 A. Electron Scan of the Anterior Portion of Schistosoma mansoni. At 25 µg/ml of oltipraz, shrinkage of the oral suckers (OS) is apparent. The opening of the canalis gynecophorus (CG) is seen. X150.

B. Dorsal Side of the Anterior Section of Schistosoma mansoni Depicting Dips and Depressions as well as Shrinkage of the Tegument. X111.

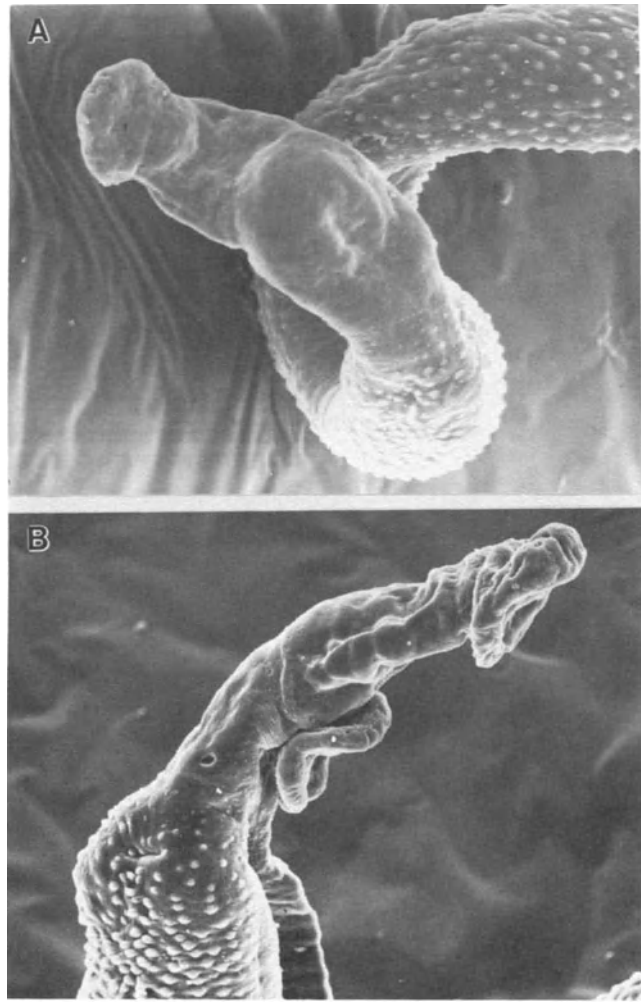

Figure 6 Head Region of Schistosoma mansoni Exposed to 1000 µg/ml (A) and 1500 µg/ml (B) of Oltipraz. Note as the concentration of oltipraz increases, more damage is apparent. X150.

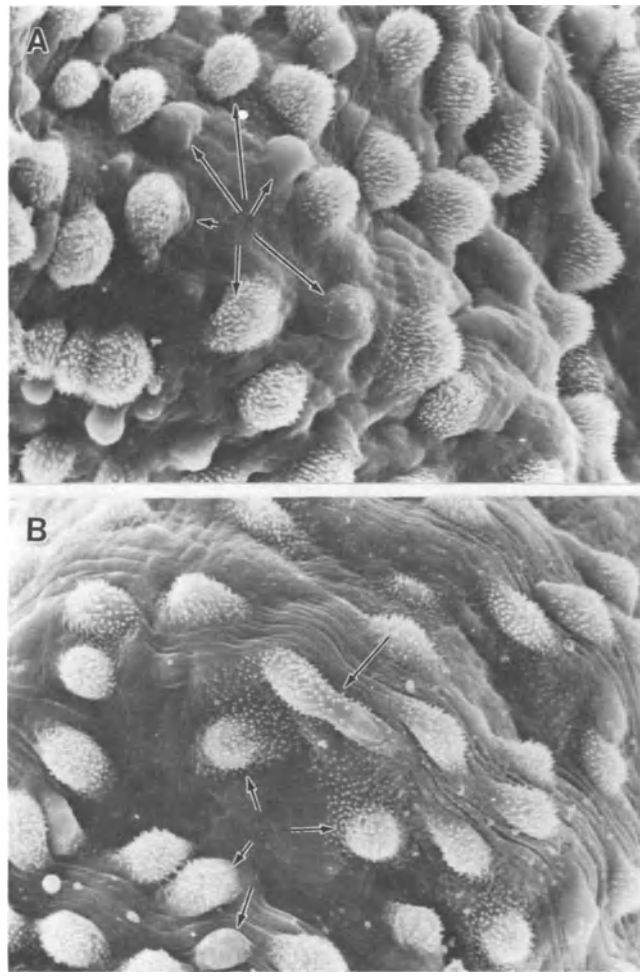

Figure 7 High Magnifications of the Anterior End (Lower Neck Region) of the Treated Worm Revealing Spine and Spineless Bosses of Irregular Sizes, Shapes and Distribution Indicated by Arrows. A. 25 µg/ml X813 and B. 50 µg/ml X815.

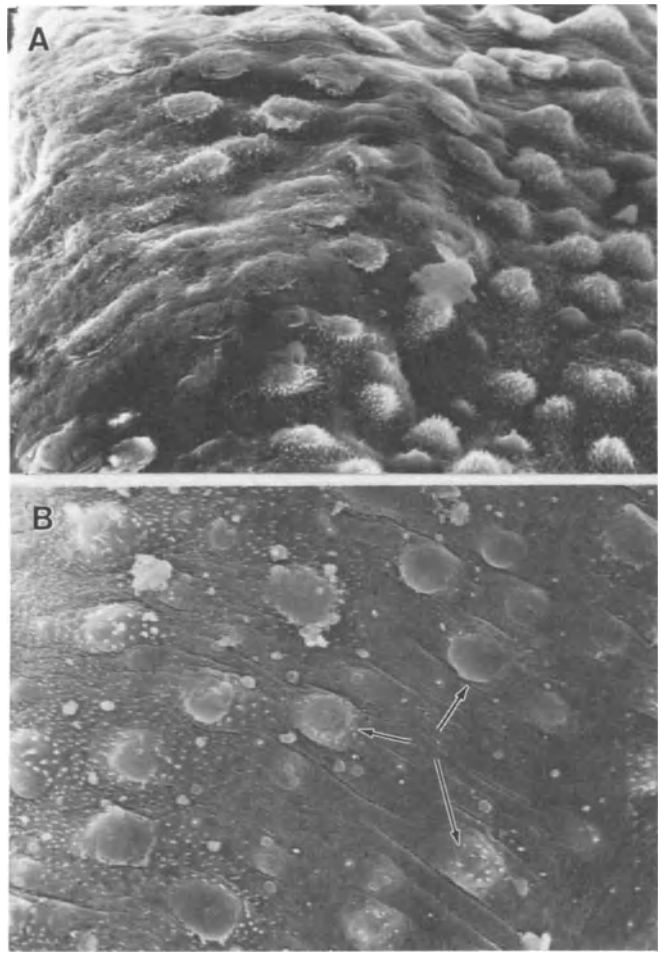

Figure 8 A. Surface Tegument Showing Irregular Shapes and Distribution of the Bosses. Note: Sloughing of the Tegument is Apparent. X715.

B. In Some Areas of the Tegument, Spines Exist Around the Base of the Bosses (Arrows). X735.

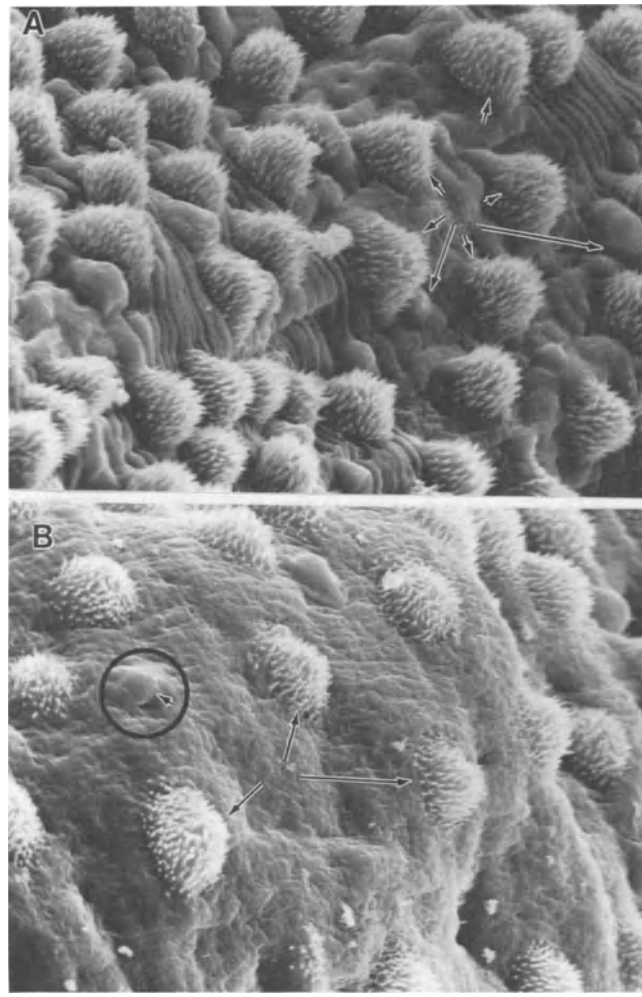

Figure 9 A. High Magnification of the Mid-body Region Exposed to 25 µg/ml Oltipraz. The bosses appear more pointed and erect (arrows) and the tegument appears folded. X799.

B. At 50 µg/ml of Oltipraz the Bosses in Some Areas of the Tegument Are More Widely Distributed on the Surface Tegument (arrows). The spineless bosses seem to have taken unusual shapes (circle arrow). X845.

bosses in other areas of the tegument were more widely distributed on the surface. Incorporation of 1000 µg/ml and 1500 µg/ml of oltipraz revealed a ruffled surface tegument and the bosses showed the appearance of moving inwards (Figures 10A-B).

Spines located on the posterior end of the parasite appeared irregularly distributed on the surface after exposure to 25 µg/ml and 50 µg/ml of the drug (Figures 11A-B). Worms treated with 1000 µg/ml and 1500 µg/ml exhibited the presence of depression and shrinkage of the tegument (Figure 12A). In some instances the tail appeared to be contracted (Figure 12B). Shrinkage and degeneration changes in the bosses were evident along the posterior end of the worm at higher magnifications (Figures 13A-B and Figures 14A-B). Utilizing x-ray microanalysis, it was not possible to detect the component(s) of oltipraz at 1500 µg/ml in the treated worms. However, other elements such as Na, Fe and Ca were detectable.

These findings are in agreement with results reported by other investigators. For example, Shaw et al. (1983) reported that praziquantel administered to S. mansoni infected mice caused tegumental damages that resulted in surface lesions. Subsequently, ultrathin sections of S. mansoni exposed to praziquantel (in vivo and in vitro) revealed intensive vacuolation in specific sites of the tegument (Andrews 1981; Becker et al., 1980). Verheyen et al. (1978) reported time-related topographical changes in mature Cysticerci of Taenia taeniaeformis induced after medication of infected mice with 250 ppm mebendazole. These alterations included disappearance of microtribes and progressive degeneration of the tegument exhibiting an irregular surface consisting of grooves, holes and crater-like structures.

The changes in the tegument seem to depend primarily on the concentration as well as the length of time the parasites were exposed to the drug. Schistosoma mansoni exposed to low doses of oltipraz, 25 µg/ml, for more than 12 hrs consist of severe surface tegumental damage (data not shown). Jones et al. (1989) reported similar findings after exposing C. fasciolaris to low (25 µg/ml) and high (1500 µg/ml) concentrations of oltipraz.

The present results suggest that S. mansoni worms incubated in 25 to 1500 µg/ml oltipraz incubation for various time intervals causes damage to the surface tegument. However, further ultrastructural studies are underway to better understand the mechanism of attachment and the transport of olitpraz into the tegument of S. mansoni. In addition, biochemical studies will also be employed to determine

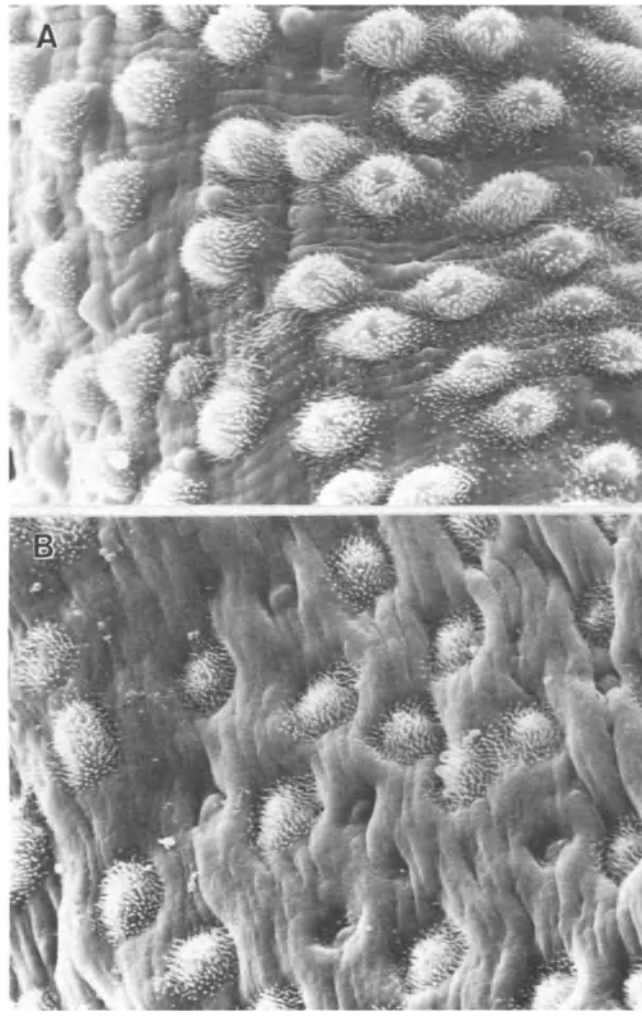

Figure 10 A. Incorporation of 1500 µg/ml of Oltipraz Showed a Ruffled Surface. X749.

B. At 1500 µg/ml Structural Changes of the Surface Tegument are More Pronounced. The bosses appear to be moving inward on the tegument. X700.

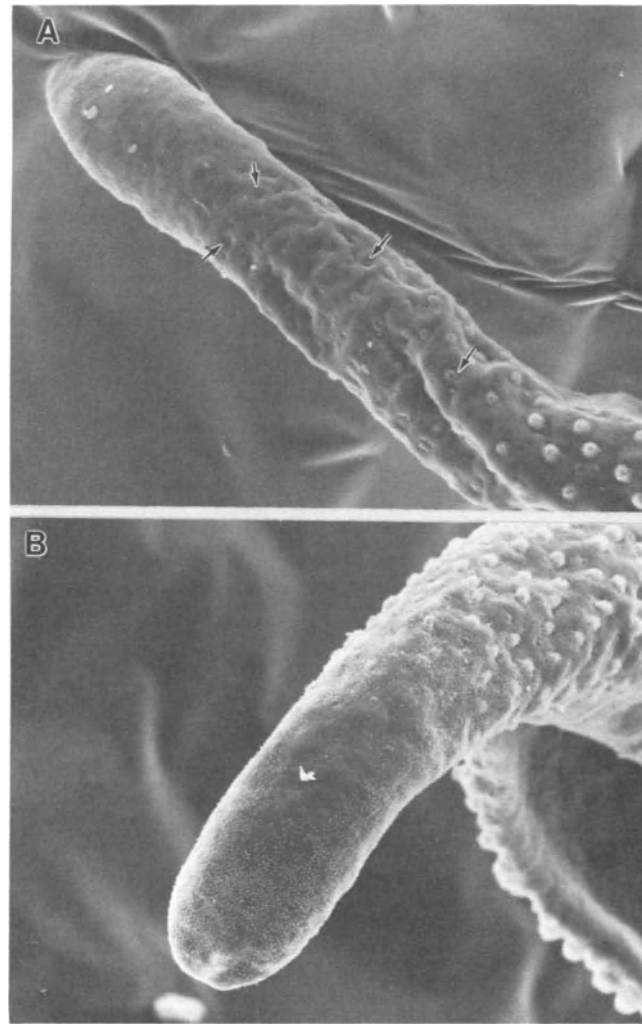

Figure 11 A. Posterior End of the Treated Worm. In the presence of 25 µg/ml of oltipraz the tail of the worm appears to become extended, and depressions are evident on the surface (arrows). X130.

B. 50 µg/ml of Oltipraz. Spines were randomly distributed. X319.

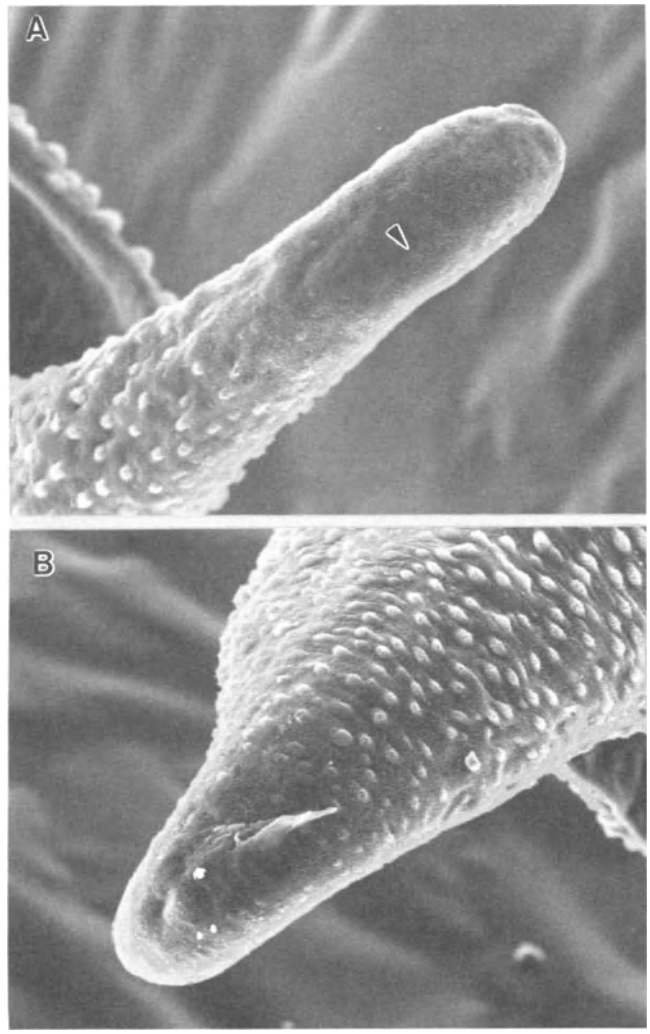

Figure 12 A. 1000 µg/ml of Oltipraz. Depression and Shrinkage is Apparant (arrows). X837.

B. 1500 µg/ml of Oltipraz. The tail of this worm appeared to become shortened. Bosses can be seen more closely to the end of the tail. X309.

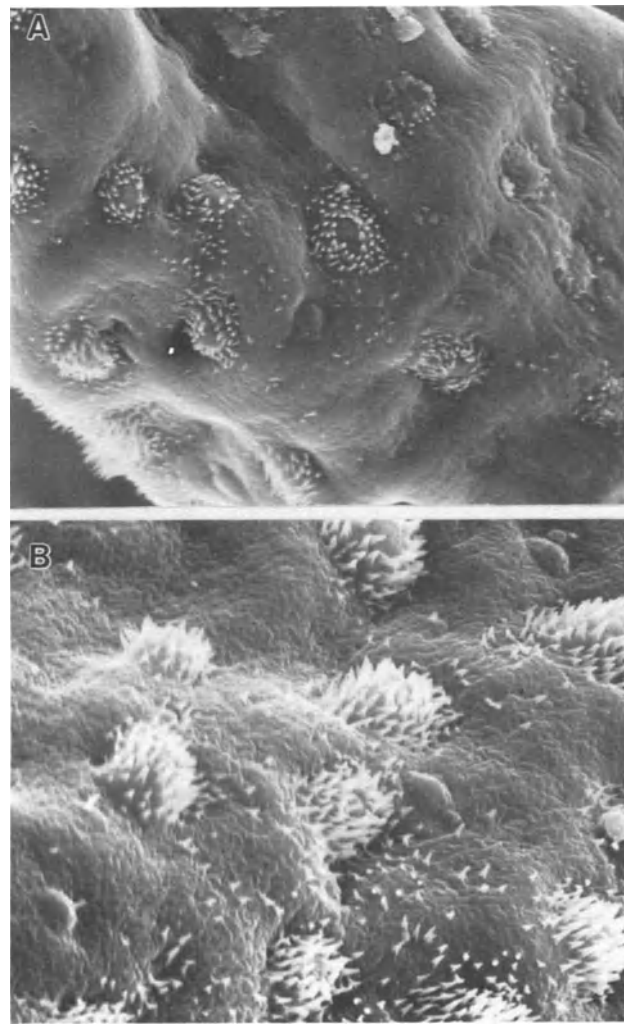

Figure 13 A. High Magnification of the Posterior Section of the Worm Exposed to 25 µg/ml of Oltipraz. Note: The depression of bosses into the tegument. X1.152.

B. High Magnifications of the Posterior Section of the Worm Exposed to 50 µg/ml of oltipraz. The tegument appear ruffled and the bosses are irregularly shaped. X2.004.

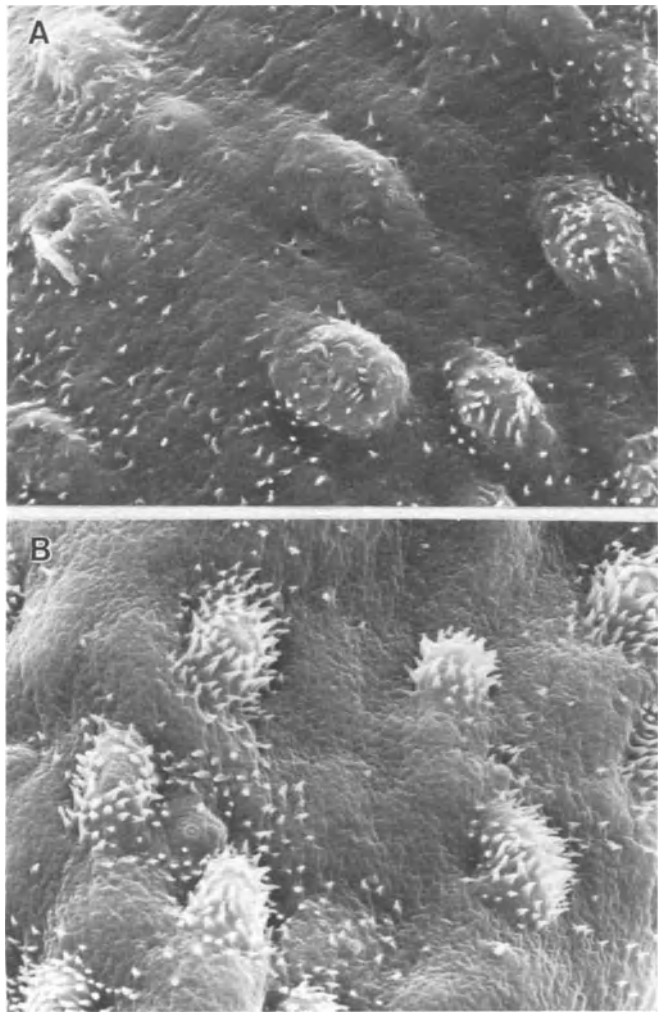

Figure 14 A. At 1000 µg/ml of Oltipraz the Tegument Appear Devoid of Spines. X2.016.

B. At 1500 µg/ml of oltipraz, note the Folding and Irregularity of the Bosses and Surface Spines. X2.007.

µg/ml exhibited the presence of depression and shrinkage of the tegument (Figure 12A). In some instances the tail appeared to be contracted (Figure 12B). Shrinkage and degeneration changes in the bosses were evident along the posterior end of the worm at higher magnifications (Figures 13A-B and Figures 14A-B). Utilizing x-ray microanalysis, it was not possible to detect the component(s) of oltipraz at 1500 µg/ml in the treated worms. However, other elements such as Na, Fe and Ca were detectable.

These findings are in agreement with results reported by other investigators. For example, Shaw et al. (1983) reported that praziquantel administered to S. mansoni infected mice caused tegumental damages that resulted in surface lesions. Subsequently, ultrathin sections of S. mansoni exposed to praziquantel (in vivo and in vitro) revealed intensive vacuolation in specific sites of the tegument (Andrews 1981; Becker et al., 1980). Verheyen et al. (1978) reported time-related topographical changes in mature Cysticerci of Taenia taeniaeformis induced after medication of infected mice with 250 ppm mebendazole. These alterations included disappearance of microtribes and progressive degeneration of the tegument exhibiting an irregular surface consisting of grooves, holes and crater-like structures.

The changes in the tegument seem to depend primarily on the concentration as well as the length of time the parasites were exposed to the drug. Schistosoma mansoni exposed to low doses of oltipraz, 25 µg/ml, for more than 12 hrs consist of severe surface tegumental damage (data not shown). Jones et al. (1989) reported similar findings after exposing C. fasciolaris to low (25 µg/ml) and high (1500 µg/ml) concentrations of oltipraz.

The present results suggest that S. mansoni worms incubated in 25 to 1500 µg/ml oltipraz incubation for various time intervals causes damage to the surface tegument. However, further ultrastructural studies are underway to better understand the mechanism of attachment and the transport of olitpraz into the tegument of S. mansoni. In addition, biochemical studies will also be employed to determine specifically the component(s) that are involved with oltipraz-induced destruction of the tegument.

SUMMARY

This paper describes the in vitro effects of the anti-schistosomal drug, oltipraz, on the surface tegument of Schistosoma mansoni. Oltipraz administration at low and high concentrations (i.e., 25, 50, 150, 250, 500, 1000 and 1500 micrograms) in relation to time (i.e., 30, 60, 90 and

150 minutes) causes significant changes in the surface tegument. These changes include: progressive necrosis of the oral and ventral suckers, irregular shaped and involution of both the spine and spineless bosses. Schistosoma mansoni exposed to 25 µg/ml of oltipraz for 30 min to 60 min appear to have very little tegument damage in relation to Schistosoma mansoni exposed to the drug for longer periods (12 hrs). Extensive tegumental damage was apparent when the worms were exposed to 25 µg/ml for more than 12 hrs. These damages include: pronounced shrinkage and necrosis of the entire surface tegument. Finally, more detailed ultrastructural and biochemical studies are underway to better understand the mechanism of action of oltipraz on the tegument of S. mansoni.

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